Cell Homing of Immature Permanent Teeth Implanted in Rodents During Pulp Regeneration

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

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Zürich, 2016

'Happiness does not depend on what you have or who you are. It solely relies on what you think.'

-Buddha-

Zusammenfassung

In der regenerativen Endodontie wird experimentell das sogenannte „Cell homing“ Konzept bei jungen, permanenten Zähnen zur Zahnwurzelbehandlung angewendet, um eine gesunde Zahnpulpa zu generieren, die in der Lage ist, das Wurzelwachstum fortzuführen. Im Gegensatz zur bisherigen Behandlungsmethode, bei der der Pulparaum mit nicht abbaubaren Materialien gefüllt wird, werden diese im neuen Verfahren durch biologische Gerüste, kombiniert mit Wachstumsfaktoren ersetzt. Die Prozesse einer solchen Behandlung sind bisher noch nicht vollständig verstanden, da sie von verschiedenen Faktoren abhängig sind. Ausserdem ist es wichtig die Biologie der Zahnpulparegeneration besser zu verstehen, um das Einwandern von Zellen und Geweben in den Zahnwurzelkanal verbessern zu können. Daher war das erste Ziel dieser Dissertation im Rattenmodel einen geeigneten Einbettungsort für menschliche Zähne zu finden, um damit der klinischen Situation besser zu entsprechen. Mit sorgfältig vorbereiteten und Fibrin-Gel gefüllten jungen, menschlichen Zähnen, haben wir herausgefunden, dass direkt auf dem Schädelknochen der beste Ort ist, um die Zähne einzubetten. Ausserdem haben wir das Einwandern von Zellen mit Stammzell-ähnlichen Eigenschaften in den Zahnkanal untersucht. Während bereits ohne Verwendung von exogenen Stammzell- oder Wachstumsfaktoren ein neues, Zahnpulpa-ähnliches Gewebe gebildet wurde, war ein weiteres Ziel dieser Dissertation, den Vorgang des Einwanderns der Zellen zu beschleunigen und den regenerativen Prozess damit in kürzerer Zeit zum Abschluss zu bringen. Unsere Untersuchungen zeigten, dass durch den Stammzellfaktor, (SCF), das Einwandern von Zellen beschleunigt wird und dadurch die Reifung des entstandenen Gewebes schneller voranschreitet. Auch wenn für eine erfolgreiche klinische Anwendung weitere Experimente von Nöten sind, zeigen diese Ergebnisse bereits, dass mit dem „Cell

homing“ Konzept präklinisch gute Ergebnisse erzielt werden können. Künftige Arbeiten an zellbasierten Modellen könnten dazu dienen, um auf molekularer Ebene zu untersuchen, wie die eingewanderten Zellen innerhalb des Zahnwurzelkanals zu Pulpa-ähnlichem Gewebe heranreifen. Ausserdem müssen klinische Versuche durchgeführt werden, um diese Ergebnisse aus dem Tiermodell, in die zahnklinische Praxis zu überführen.

Abstract

In regenerative endodontics, the so-called cell homing approach in immature permanent teeth is a novel root canal treatment approach that attempts to regenerate a functional pulp by applying a scaffold and morphogens rather than a mere prosthetic material in the root canal system. The outcomes of this treatment are still uncertain, as they depend on various factors. Accordingly, there is a need for better understanding the biology behind pulp regeneration as well as an improving tissue ingrowth into the pulp space. The primary objective of this dissertation was to find an optimal site for human tooth specimen implantation in the rat model, so that the clinical situation could be mimicked. We found that on top of the calvarial bone was the place for implanting the immature human teeth, which were carefully prepared and filled with fibrin gel. Migration of cells with MSCs-like properties into the root canal was also studied. Dental pulp-like tissue was efficiently formed without the application of any exogenous stem cells or growth factors. Another aim was thus to accelerate the migration of cells with MSCs-like properties and hence improve the regenerative process. SCF, used as a chemotactic agent in this study, enabled cell recruitment and successively accelerated tissue maturation. These results suggest that cell homing is indeed feasible yet requires more investigations prior to successful clinical application. Future studies in an advanced cellular function may explain how the migrated cells transform intracanally to pulp-like tissue. Clinical trials will be necessary to investigate the possibility of translating these results from the animal model into clinics.

Overview

Chapter I Introduction

A summary of pulp-dentin biology relates to this project. Novel treatment trend and strategeis of regenerative endodontic as well as principle of cell homing concept were introduced here.

Chapter II Implantation site impacts tissue ingrowth

Study model in animal was established following observation of specimen embedding area between calvaria and dorsum. 3-mm tooth sections loaded with fibrin gel were placed differently. Calvarial specimens showed greater tissue development compared to the dorsal area. Accordingly, we decided to transplant our specimens on top of rat calvaria.

Chapter III Characterization of cells occupying the pulp space of ectopically transplanted human teeth in rodents.

Following pulp-like tissue formation even though no exogenous stem cells delivered, the cell population was characterized in this chapter. Fibrin gel was filled either in the root sections or the silicone tubes (control). Intracanal cells demonstrated more MSCs phenotype which can differentiate to odonto/osteoblast and adipocyte while intra-tubule cells were not. In addition, gene expression as well as immunohistochemistry of DSP and BSP verified the differential potency of the migrated cells to pulp progenitor cells.

Chapter IV Stem cell factor: a promising chemokine for acceleration of pulp regeneration.

Stem cell factor (SCF) was combined with fibrin hydrogel to accelerate regenerative process. This molecule affected not only cell migration but also cell proliferation and cell differentiation *in vitro*. Its effects differently performed when transferring to animal model. Tissue ingrowth was greatly formed within 6 weeks and, in a longer period, more development of pulp-like tissue characteristic.

Chapter V General discussion and conclusion

Conclusion from each chapter was linked together to find the relationship from motivation of the work to all experimental results. Possible future works are also suggested in this chapter with emphasis towards clinical translation.

Acknowledgements

All of the work presented here was conducted in the oral bioengineering and biotechnology laboratory at the University Hospital Zurich and Center of Dental Medicine, University of Zurich. All projects and associated methods were approved by the University of Zurich's Research Ethics committee.

Foremost, I would like to thank Prof. Franz E. Weber for his supports to this project and his valuable given opportunity to me during the course of this PhD, as well as his countless time on continuous guiding and greatest incentive.

I would also like to express my sincere gratitude to Prof. Matthias Zehnder for an incredible experience through all the coaching. His guidance and enthusiasm were crucial for me to pursue a PhD at University of Zurich.

I am especially thankful to Prof. Johannes Loffing for his contribution and remarkable disposition of my study. Many memorable thanks go to Prof. Klaus Grätz for the admiration to my PhD, Prof. Hans Ulrich Luder for confirming our histology interpretation at the beginning, Dr. Raphael Patcas for his generosity in providing the teeth and Dr. Stéphane Simon for his interests in this project.

Additional acknowledgements to animal caretaker Flora Nicholls and her team, Thea Fleischmann, Miriam Lipiski and Mareike Sauer, are invaluable, also technical support from Helga Lüthi-Schaller, Sanya Hintz, Beatrice Senar and Dr. Philipp Lienemann. As same as histological devices and flow cytometry analysis were supported from ZMB (Center of microscopy and flow cytometry at university of Zurich). And a kind gift of human mesenchymal stem cells received from Dr. Martin Ehrbar.

I owe my deepest gratitude to my colleagues who help me solving many struggle problems. Many skills were trained by Dr.Chafik Ghayor, Dr.Lindsay Sulzer, Alexandre Tschubukov,

Anna Perez, Bebekka Gjokski and Yvonne Bleomhard as well as Barbara Siegenthaler both who are also my mentality supporters. It is grateful that we share our moment together.

It is also a grateful support from swiss government scholarship and dental association of Switzerland for providing a funding to conduct this project.

Last but not least, I would like to thank my colleagues in Bangkok, Assoc. Prof. Vanida Sangalangkarn, Prof. Jirapun Punwutikorn and Prof. Aurasa Waikakul that encouraged me to start my PhD. Finally, it is always a meaningful support that my parents, my sisters and brother, my boyfriend and my friends both here in Switzerland and in Thailand, love and care.

Zurich, 22 September 2015

Nisarat Ruangsawasdi

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Chapter 1

Introduction

Root canal treatment of immature teeth with incomplete root formation may potentially require removing the diseased pulp (pulpectomy) and apexification. Recently, regenerative endodontics has emerged as an alternative treatment for immature teeth with infected/necrotic dental pulps. The objective of this treatment is to regenerate a functional pulp that maintains pulp-dentin interactions [1]. The root and dentin of immature teeth do not continue to develop after conventional treatments, whereas the completion of dentin growth accompanied by apical closure could be enabled by regenerative endodontics.

A plethora of potential treatment strategies have been suggested after the discovery of various stem cell sources in the oral cavity, especially the apical papilla tissue, which surrounds the root apex of immature teeth and contains mesenchymal stem cells [2]. Accordingly, clinicians have attempted to induce the apical papilla to bleed and thus infiltrate the previously cleaned and disinfected pulp space with pluripotent cells [3]. The outcomes, however, are still unpredictable with a substantial number of reported failures [4]. Although some researchers support stem cell utilization for tissue engineering [5], clinicians hitherto omit this application to patients due to difficulties and tissue loss during the harvest of potential stem cells, as well as the uncertainty about the effects to the host [6]. Therefore, the stem cell-based treatment still remains at the laboratory level.

In the near future, the so-called cell-free approach appears to be the more reliable and more foreseeable option. This concept, which has been coined cell homing, is to recruit host stem cells into a specified area using molecular cues to appropriately direct cell differentiation. Given the evidence of various stem cell sources in the oral cavity, this approach is easier

translated into clinical practice. There is no need for stem cell harvesting or any certification. Only scaffold materials and growth signaling molecules are required to initiate host stem cell migration to the pulp space [6].

Although cell homing has a higher chance for clinical translation, there are still several hurdles to overcome. The optimal scaffold is still unknown and the signal cues necessary for pulp regeneration are also ambiguous. Moreover, pulp tissue is a complex structure containing four different regions of soft connective tissue. It needs to integrate with the hard mineralized dentin and is composed of a variety of cell types. Regeneration of new functional pulp tissue might require the optimal combination of scaffold and signal cues, which can attract stem cells that should differentiate into a specific cell type at the appropriate location. Various aspects should be investigated so that we can apply advanced biotechnology in regenerative endodontics and find opportunities for clinical applications.

1.1 The pulp-dentin complex

Dentin and pulp tissue have a cooperative relationship. The pulp, which lacks an epithelial barrier against the outside, requires a protective function provided by dentin, while cells in the pulp maintain dentin vitality. They are intrinsically interlinked and are mutually essential. This is why pulp and dentin together have been referred to as pulp-dentin complex.

1.1.1 Dentin structure and its composition

A tubular structure of dentin is composed of 70 wt% inorganic matrix, and 30% organic matrix with 10% water. The crystalline hydroxyapatite is doped with other minerals, the major inorganic substituents being carbonate, sodium and magnesium, which are located on and among the collagen fibers. The organic matrix mainly contains collagen type I, which is embedded in a non-collagenous matrix including proteoglycans, dentin phosphoprotein, acidic protein and numerous types of growth factors [7].

In early tooth development, odontoblasts secrete dentin matrix to form mantle dentin prior to the growth of predentin, which initiates calcification. Initially, collagen type III is secreted followed by the production of collagen type I, to develop a scaffold for dentin. Non-collagenous proteins such as DPP, DSP, OCN and BSP can bind to this matrix and initiate the mineralization process at the dentin interface [7]. The conversion is regulated by the action of growth factors including BMP2, TGF-β and bFGF. The dentin preserves these proteins as a matrix and the growth factors remain until they are released [8, 9].

The types of dentin secreted by odontoblasts are mainly primary dentin and secondary dentin. Tertiary dentin, which is created to respond to noxious stimuli, will be mentioned in the subsequent section. While primary dentin is formed during tooth development, the secondary dentin is secreted continuously throughout human life. During odontogenesis, odontoblasts secrete primary dentin in order to form a functional tooth. When the cusps of opposing teeth contact each other, secondary dentin begins to be formed, yet at a slower rate than the primary dentin. Although both types of dentin are similar in composition, their morphology is slightly different as the secondary dentin has a more accentuated s-curve of the tubules than the primary dentin [10].

The tiny tubules of dentin are filled with the odontoblastic process surrounded by dentinal fluid, which can move freely in the dentinal tubule. Mechanoreceptors on nerve endings subjacent to the odontoblast layer can sense the fluid movement [11]. This stimulation, if it continues over the time, will alter the secretion activity of the odontoblasts in order to limit fluid movement and pain. In view of that, the communication between the dentin and pulp is facilitated by dentinal fluid and odontoblasts [10].

1.1.2 Dental pulp

The dental pulp is a loose connective tissue that, in addition to providing the cells necessary for tooth development, has various postnatal roles serving multiple biological functions. Organic compounds of the surrounding mineralized tissue are supplied with nutrients from the pulp, while the nerves sensing chemical and physical changes occurring within the pulp and dentin help to prepare a defensive response to harmful stimuli, e.g. by creating tertiary dentin.

The physical structure of the pulp is reflected through the biochemical components of its extracellular matrix (ECM). Varying sizes of collagen fibers, mainly types I and II, align to become the ground substance. Water is present in the pulp matrix accounting for 75% and the organic elements account for 25%. The non-collagenous proteins such as proteoglycan (decorin, biglycan, versican, syndecan, tenascin and fibronectin) and glycosaminoglycans (dermatan sulfate, heparan sulfate, chrondoitin sulfate and hyaluronan) are hydrated in the collagen matrix and create an ECM with distinctive gel-like properties [10].

There are four different pulp areas, each of which contains different cells and tissue components. The innermost area is the central pulp zone. At the periphery of the pulp, the odontoblastic layer connects to the predentin. The cell-free zone and the cell-rich zone are located under the odontoblastic layer (Fig. 1). Main blood vessels and lymphatic vessels, as well as large nerve trunks at the central pulp zone, connect the tooth with the body and transport nutrients to the pulp. Adjacent to the core, the cell-rich zone contains a high density of cells and is rich in capillary plexus. This area is thought to conserve undifferentiated mesenchymal cells necessary for pulp repair and regeneration. The cell-free zone is the next area locating towards the dentin and forms after completion of dentinogenesis. Small capillary and nerve plexus, which are used to support young odontoblasts, move to the area

beneath the odontoblastic layer. Capillaries also continue to bring nutrients to mature odontoblasts while nerves transfer neuromodulators from them [12].

A variety of cells within the pulp is embedded in the ECM. The major cell type is fibroblasts, which synthesize and secrete the collagen matrix while odontoblasts are specialized cells acting as a mediator between pulp and dentin. Mesenchymal stem cells are also found in a vast number within the pulp where various progenitor cell niches have been identified [13, 14]. Other cells that can be found include dendritic cells and immune cells such as macrophages, lymphocytes and neutrophils [7].

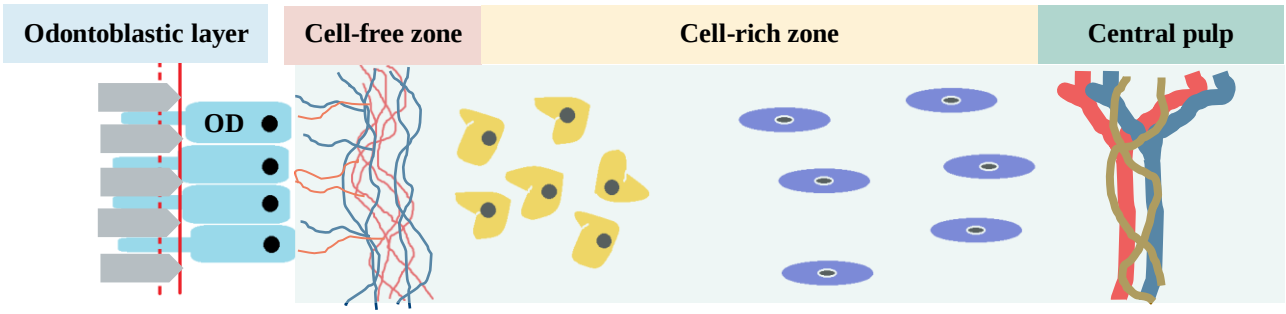

Fig. 1 Different pulp zones. The innermost area is the central pulp where main blood and nerve trunks supplement fibroblast and undifferentiated cells within the *cell-rich zone*. Odontoblasts (OD) form the outermost zone, the *odontoblastic layer*, which is supported by small blood vessels and nerve plexus from the *cell-free zone*.

Odontoblasts are formed at the early stage of tooth development and are maintained in the healthy pulp tissue to repair impaired dentin. There are several morphologies depending on the stage of maturation and the area where they are localized; from a squamous spindle shape at the root apex to cuboidal cells at the level of the cemento-enamel junction, and finally more coronal to large columnar cells. In some areas, odontoblasts might be present in a monolayer while a pseudostratified pattern can be identified in a highly regenerative area

[15]. The secretory activity of the odontoblasts is important for dentin formation in early tooth development. Nevertheless, it can be up-regulated when the dentin needs to be repaired. The other cell types, like immune cells, stem cells and progenitor cells, also participate in this pulp-dentin complex to maintain a biological balance. Stimuli from bacteria or any traumatic injuries lead to the recruitment of those cells via pulp and dentin interactions. When the circumpulpal dentin is destroyed due to bacterial activities, the cells within the pulp can sense and respond through immunocompetent odontoblasts to prevent such stimuli from compromising pulp vitality. Immune cells from the blood stream accumulate at the area of invasion to kill bacteria and cause inflammation, while stem cells and/or odontoblast progenitor cells produce dentin from the inside to prevent the invaders from accessing the pulp [7].

1.2 Biology of pulp involving injury, repair and regeneration

The tooth has the capacity to sense and defend against environmental challenges to maintain its vitality. Dental caries, trauma and erosion can affect pulpal health. A cellular response within the pulp can respond to those challenges. Odontoblasts are the key cells responsible for the interplay between the regenerative response and inflammation, whereas other pulp cells support these processes.

1.2.1 Tertiary dentinogenesis

Tertiary dentin is pathology-driven dentin. It is formed under the circumstances of tooth injury to isolate the living pulp tissue from external irritants. There are two types of tissue formation: reactionary dentin and reparative dentin. They are created differently depending on the extent and severity of the irritation and its progression rate.

As long as the primary odontoblasts are present, reactionary dentin is produced to preserve the tooth’s vitality. Slow and minimally invasive injuries are not harmful to odontoblasts

beneath the caries lesion. This low-grade response can stimulate odontoblasts to synthesize sclerotic dentin while circumpulpal dentin is produced during an accelerated dentinogenesis process [16]. The injured odontoblasts lay down sclerotic dentin in the lumen of dentinal tubules to reduce the permeability of dentin and to prevent the diffusion of bacterial components and other irritants. Circumpulpal dentin is deposited at the dentin-pulp interface to increase the thickness of the protective shield.

The formation of tertiary dentin involves many bioactive molecules such as the TGF-β family (1 and 3) and the BMP 2, 4, and 7. They penetrate through the dentinal tubules upon being released during the demineralization of dentin by bacterial acid and interplay with ECM proteins such as fibronectin to stimulate odontoblasts to secrete different types of dentin [17]. The amount of production is proportional to the number of growth factors released from dentin, and is reversely related to the thickness of dentin located between the lesion and odontoblasts [7].

In the case of severe caries progression, odontoblasts are destroyed and cells from the subodontoblastic area take their place. These undifferentiated cells can replace odontoblasts to regulate the inflammation and the recovery of the pulp. Those cells might be odontoblast daughter cells originating from the dental mesenchyme, which lack the induction signal from the basement membrane during tooth development. Alternatively, they might be pulp progenitor/stem cells migrating from the cell-rich zone [10].

The supplement cells become odontoblast-like cells as soon as they adhere to the dentinal wall and rapidly secrete the extracellular dentin matrix composed of collagen types I and III, and fibronectin [18]. The mineralization begins to form fibrodentin, which contains fewer minerals. The rapid formation causes some cells to be trapped within the tissue. This bone-resembling dentin is called osteodentin and the trapped cells are termed osteodentinoblasts. Once the barrier is created, pulp cells can slowly generate tubular dentin from the

odontoblast-like cells induced by TGF-β and fibronectin provided by fibrodentin [19]. The reparative dentin contains large amounts of collagen type III and fibronectin in addition to collagen type I [7].

1.2.2 The defensive responses

The dentin-pulp complex responds to bacterial infections and other pathological triggers by involving multiple cellular signals. These mechanisms control the inflammation and simultaneously repair the damaged tissue. Various processes have been known to happen simultaneously during pulp injury and regeneration. The primary phase cells are odontoblasts located at the periphery of the pulp tissue. Toll-like receptors on the odontoblasts will firstly detect and mount the pulpal defense. The balance of activation by the host and bacteria through the p38 MAP-kinase pathway will potentially regulate the links between the early stage of infection and the repair process, which is in turn regulated by the odontoblasts [20]. These cascades subsequently stimulate other pulp cells including fibroblasts, endothelial cells and resident immune cells to the site of infection. The immune cells are then present following the secretion of many cytokines such as interleukins and TNF-α. The profile of these proinflammatory molecules and processes affect the survival of healthy pulp and the regenerative events [21]. Once the infection is under control and the inflammation is manipulated successfully, dental pulp cells will switch to the reparative phase.

While bacterial elements directly trigger pulp inflammation, the acid secreted from them also causes a release of non-collagenous proteins from dentin. As dentin is a reservoir of bioactive molecules, it is conceivable that the growth factors and cytokines within it direct cellular signaling. The first role of tissue factors released from dentin is to recruit immune cells and support their bacterial killing mechanism. Secondly, the matrix components are in direct contact with odontoblasts, which are triggered in reparative and regenerative circumstances. Among a cocktail of growth factors, the TGF-β superfamily has the most impact on cellular

reactions. It stimulates differentiation and the secreting function of odontoblasts. These dentin-mediated activations are generally wide-ranged and are unlikely to be area-specific [22].

Stem/progenitor cells and immune cells together orchestrate the response to deeper pulp injury and regeneration. For example, SDF-1 ligand and CXCR4 receptor association has been reported on both immune and regenerative cells as well as SCF ligand and c-kit receptors [23-25]. This implies that both cell types are sequentially involved. Following the immune cells, to regulate inflammation, stem/progenitor cells start to secrete dentin-like tissue. Furthermore, it is likely that dentin matrix components also influence cell migration, proliferation, and differentiation in a concentration-dependent manner. At low dentin derivative concentrations, the proliferative rate of the pulp cells will increase, whereas a higher concentration will retard cell proliferation [7]. However, the cell number is not necessarily representative of whether the proliferated cells can contribute to the repair process. In fact, the growth factors localized within the dentin matrix are sufficient to recruit stem cells and induce odontoblast differentiation [26].

Various growth factors and cytokines have been reported to contribute to odontoblast differentiation. Particularly, TGF-β1 within dentin seems to guide the stem cells residing in the dental pulp to differentiate to odontoblast-like cells during reparative dentinogenesis, and it notably contributes to the secretion of tertiary dentin through the p38-MAPK pathway [18, 21]. It is also surmised that pro-angiogenic and neurotrophic factors present in the dentin matrix play a role in revascularization and neurogenesis within the dental pulp after the injury [7].

1.3 Novel trend in endodontic treatment

Endodontic treatment is needed when the defense process within the pulp tissue fails to protect against invading bacteria. As the protective system collapses, inflammation spreads

throughout the whole pulp space, finally resulting in tissue necrosis. The main goal of root canal treatment is thus to remove the infected pulp and subsequently seal the tooth to prevent further ingress of oral bacteria and the thus-resulting inflammatory reactions in the tissues surrounding the root apex.

The contemporary treatment is not always the best option, particularly in regard to contemporary ways to fill cleaned and disinfected root canal systems. Root canal fillings with gutta-percha and a sealer certainly suit the fully developed permanent tooth, whereas failures occur frequently when using this method to treat the immature tooth [27-29]. The poor mechanical characteristics of young permanent teeth do not only cause trouble during the endodontic procedures, but also affect sustainability after the treatment [30].

A novel attempt has emerged over the past decade to regenerate a functional pulp in formerly infected immature teeth, i.e. immature teeth with apical periodontitis. A classical study by Calvin Torneck *et al.* showed that connective tissue could be formed in the emptied space of cylindrical tubes in rodents. Although the growth was less extensive when the tube's lumen was too long or too narrow, the study inspired the possibility of tissue in-growth into the limited area of the pulp space [31]. The concept was later transferred to human permanent teeth whereupon bleeding was induced, allowing fibrous connective tissue, as well as in some instances cementum-like tissue, to also form in the root canal [32]. However, these early reports were on mature teeth with fully formed roots. Tissue ingrowth only occurred in teeth containing a vital pulp stump, not counterparts that were fully necrotized and had a periapical lesion. Nevertheless, these studies together with the identification of stem cells in the apical papilla tissue supported the idea to induce pulp regeneration in immature permanent teeth instead of restoring them with synthetic materials [2, 23].

The first successful case, which was perhaps performed accidentally, was reported in 2001 in which, after an antibiotic paste was left in an infected immature premolar for an extended

period, the radiographic image of the treated tooth revealed an increase in root length and thickness as well as a reduction in pulp space [34]. These outcomes triggered the application of a new therapeutic concept to achieve successful pulp revascularization, which is achieved by intentional bleeding into the formerly disinfected root canal [35]. From the moment that this guideline was proposed to the endodontic field, a multitude of case reports and case series have gradually been published [36].

More recent studies, however, reported negative outcomes [37, 38], especially when the infection reached beyond the root canal. These incidents may have occurred because the apical papilla and its potential stem cells were already destroyed. Therefore, the major challenge in regenerative endodontics is pulp regeneration in the absence of a vital apical papilla, which is also the case for mature teeth. Accordingly, it is questionable whether the invading cells following the evoked-bleeding step would have the same potency as stem cells from the apical papilla to regenerate pulp. Stem cell-based approaches have thus been proposed to overcome problems of predetermination of the cells. However, a recent study revealed mesenchymal stem cell markers present on recruited cells following intra canal bleeding, not only in immature tooth [39], but also in mature teeth [40]. These findings strongly suggest differentiation potency of the migrated cells for the reconstruction of dental pulp even without the application of stem cells.

Despite reports on failure of regenerative endodontics [5], the treatment has significantly improved clinical results in immature permanent teeth. Compared to conventional root canal treatment and MTA apexification, it shows better and more reliable results and some increase in root development [34].

1.3.1 Strategies and current research in regenerative endodontics

The topic of pulp regeneration has received a lot of attention from both researchers and clinicians looking to improve the concept. The focus is on how to assemble the triad of tissue

engineering components: stem cells, scaffold and growth factors. All components are mandatory in the course of tissue engineering, but the method may differ regarding the derivation of the stem cells. Accordingly, some groups believe that the action of autologous stem cells may be insufficient for positive long-term results and propose that cells with the potency to form the dental pulp tissue should be applied [42, 43].

Most protocols of cell therapy suggested to date have combined isolated stem cells with an adequate scaffold under appropriate morphogenic signaling. *In vivo* cell transplantation studies have shown full regeneration of dental pulp-like tissue in the root canal including the deposition of new tubular dentin [5, 44, 45]. In general, the target stem cells are from one of the following niches: DPSCs, SHED, SCAP, BMSCs and ADSCs. Their manipulation normally requires retrieval and *ex vivo* expansion in the laboratory prior to use. Therefore, a protocol should follow the good manufacturing practice (GMP) guidelines, however excessive costs will make it difficult to implement in clinics [46].

In contrast, the cell-free approach has shown the most promise for clinical translation. According to reported cases with induced bleeding, the generation of a blood clot forms a scaffold for apical papilla cells to home, while other growth factors are released from platelets during the clotting cascade and are also present at the pulp-dentin interface [47]. Although the main components necessary for tissue engineering appear to be fulfilled already in this current clinical concept, it is still somewhat uncontrollable and unpredictable. Side effects such as tooth discoloration are frequent, and continued root development is not achieved routinely. Moreover, revascularized immature teeth that had to be extracted for reasons unrelated to the procedure showed that the tissue that forms in the root canal space is not pulp, but rather of periodontal origin [48]. Attempts to improve the cell homing concept thus should focus on how to enhance the potency of the scaffold, also by determining necessary signaling molecules [3].

Apart from the strategies divided by cell usage, other aspects of the treatment protocol are under scrutiny in regenerative endodontics. There is evidence that root canal irrigants and medicament may have adverse effects on new tissue formation [49]. Disinfectants like sodium hypochlorite and antibiotic root canal pastes can harm stem cells and impede revascularization in the root canal [49-51]. In contrast, bio-compatible chelating agents such as EDTA have been suggested to condition the dentin prior to cell relocation. EDTA-mediated effects include liberation of growth factors such as TGF-β1, BMP-2 and FGF-2 from the dentinal wall. EDTA thus promotes collagen-based dentin matrix directly in contact with the cells [9, 10, 22]. Likewise, a calcium hydroxide suspension has been recommended to replace the use of antimicrobial for intra root canal medication [51]. These clinical procedures, employed before bleeding activation, appear to be important regarding their impact on pulp tissue regeneration. However, they have not yet been standardized.

In summary, there are still further studies necessary to correct and improve the concept of the cell-free approach in regenerative endodontics before consistent clinical results can be reached.

1.4 Cell homing concept

Cell homing is defined as the mobilization of autologous stem cells and their recruitment into the target site [6]. In most cases, that should involve recruitment via the blood stream. However, for regenerative endodontics, cell migration from the interstitial area can occur locally, as numerous stem cell sources beyond the root canal can provide stem cells independent of the blood circulation. Therefore, the ability of stem cells to be active and to recognize the target tissue is critical for successful cell homing during pulp regeneration.

1.4.1 Cell population

It is known that various stem cell sources exist inside the root canal and around the root apex (Fig. 2), which can provide sufficient robust cells to form dental pulp tissue [52]. However, hitherto the responsible cell source during pulp regeneration is unknown.

Within the pulp space, three stem cell niches have been characterized during different stages of tooth development: (1) SHED from exfoliated deciduous teeth, (2) TGPCs from third molar tooth germs and (3) DPSCs from mature permanent teeth [53]. However, these stem cells are eradicated following the cleaning and shaping procedure of the root canal. This implies that other stem cell sources are responsible for cell homing.

SCAP demonstrated a high potency in regenerative endodontics of the immature tooth. SCAP are early progenitor cells, which are found in the cell-rich area located between dental pulp and apical papilla [53]. Compared to DPSCs, they have a greater ability to proliferate, regenerate tissue and migrate [2]. In addition, SCAP can differentiate into odontoblast secreting primary dentin, while DPSCs are prone to transform into odontoblast-like cells, which merely produce reparative dentin [54, 55]. The lacerating of the apical papilla tissue to allow SCAP movement is, however, often impossible in cases of severe pulp infection with periradicular lesions [56, 57]. Accordingly, other stem cell populations housed in oral tissues may also play a role in the regenerative process.

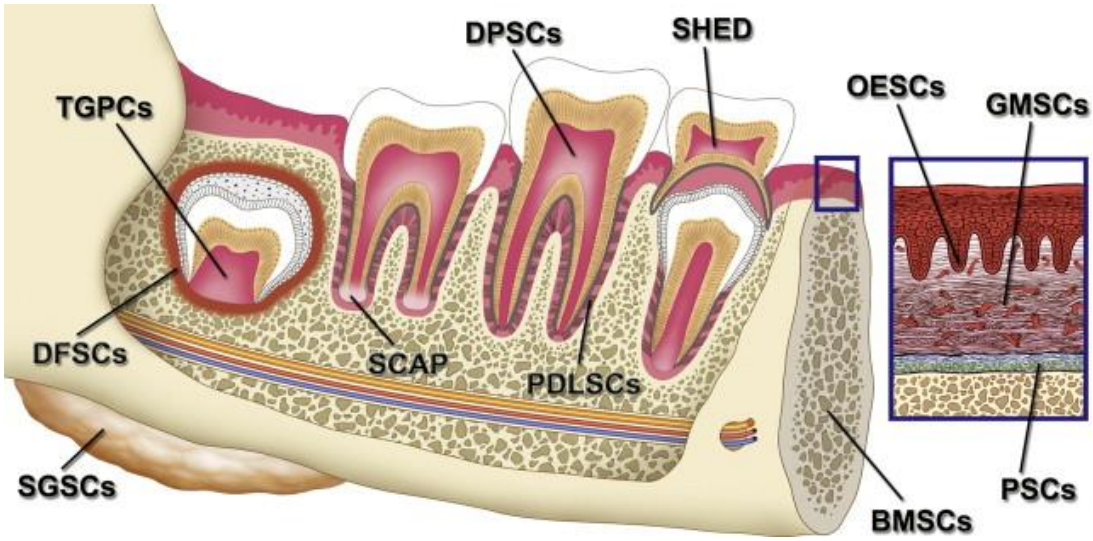

Fig. 2 Dental stem cells. Many stem cell sources are present in dental tissues. SGSCs: salivary gland stem cells, DFSCs: dental follicle stem cells, TGPCs: tooth germ progenitor cells, SCAP: stem cells from apical papilla, DPSCs: Dental pulp stem cells, SHED: stem cells from human exfoliated deciduous teeth, PDLSCs: periodontal ligament stem cells, BMSCs: Bone marrow stem cells, OESCs: oral epithelial stem cells, GMSCs: gingival-derived mesenchymal stem cells, PSCs: periosteum-derived stem cells [58].

The stem cell niches from the periodontal ligament (PDLSCs), alveolar bone (BMSCs) and periosteum (PSCs) play an important role further down the reparation process, especially when the apical papilla is eliminated by a periapical inflammation. Although these stem cells have been known for their capacity to engineer and repair cementum/bone tissue [59-61], the behavior and the immunophenotype of these cells can change after they leave their niches [62]. Once the stem cells migrate into the new environment, they should be able to form the right tissue or react in the desired manner. Likewise, PDLSCs expanded in hydrogel merely produced collagen fibers, whereas hydroxyapatite/tricalcium phosphate carrying PDLSCs revealed both cementum and collagen formation [60]. From this perspective, other stem cell sources adjacent to the root canal, like gingiva (OESCs and GMSCs), salivary glands (SGSCs) and dental follicles (DFSCs), may also be involved in pulp tissue engineering.

1.4.2 Scaffolds

An ideal scaffold for pulp regeneration requires materials that can facilitate cell movement, proliferation, cell-cell interaction and a three-dimensional spatial structure similar to ECM to home the cells. A lot of biomaterials are available for use for such purposes, including natural polymers, synthetic polymers, hydrogels and bioceramics. For pulp regeneration, the specific requirements of a material have to be taken into account in addition to its physical adaptability to create the environment for soft connective tissue regeneration, such as its handling and cost-effectiveness aspects.

A collagen sponge has frequently been used in pulp tissue engineering experiments, either with or without stem cell transplantation, because this material has similar properties to the principal substance of pulp tissue [63]. For instance, Mao's group used a collagen gel combined with a cocktail of growth factors including bFGF, VEGF, PDGF, NGF and BMP7 [64]. This growth factor doped gel was shown to recruit host cells into the root canal and to provide all the necessary elements for vasculogenesis, neurogenesis and dentinogenesis. Synthetic self-assembling peptides have also been suggested for this purpose, as they can be easily modified and handled [65].

Recently, our laboratory has suggested the use of a fibrin gel for regenerative endodontics [66]. The fibrin gel is used as a scaffold to mimic blood clot morphology and properties. It has been applied widely in the field of regenerative medicine to construct new tissues such as bone and blood vessels. For pulp tissue, it has been modified to be used at a low concentration to be injectable into the cleaned root canal space. The results of immature human teeth implanted in rats showed a full-fledged in-growth of a unique pulp-like tissue *in vivo*. In addition, fibrin gel has been clinically accepted for implantation in humans by the FDA, and therefore is highly interesting for clinical application in endodontics [47, 67].

1.4.3 Morphogens

During pulp regeneration, the action of cytokines and growth factors are important in governing tissue development during the process of cell behavior and morphogenesis. Various specialized cell types should be guided into the root canal and differentiate to functional cells. The initial step of cell homing is to recruit endogenous stem cells. SDF-1 and SCF, both chemokines, have shown a correlation and are released during stem cell mobilization [25]. Following this step, cell differentiation is driven by growth factors. These molecules can be either naturally produced or genetically engineered.

The necessary factors are thought to be involved in the formation of vessels, nerves and dentin. The platelet-derived growth factor superfamily, known as vasculogenesis and angiogenesis factors, is a group of signaling molecules that restore oxygen and nutritive supplies [68]. Neurotrophic factors, like NGF, are also involved in conducting the peripheral nerve regeneration and gradual functional restoration [19]. These are the basic elements for restoring the tissue. As the dental pulp still requires odontoblasts to continue dentin formation and to maintain the pulp-dentin complex, transforming growth factors and the bone-morphogenic protein family are the most considered factors in regenerative endodontics [69]. These can be added to the scaffold material or released from dentin by the application of EDTA in an endodontic irrigant. Regardless of the method applied, the end result should lead to the presence of functional odontoblasts at the dentinal wall.

1.5 Aims

In this study, we aimed to regenerate a fully functional pulp tissue by using the cell homing method in a rodent model. To accomplish this, three objectives were proposed as follows:

- To establish a reliable and controllable animal model with clinical relevance that can also be used to study cell homing during pulp regeneration.
- To characterize the invading cell population into the human tooth during pulp regeneration in this model.
- To define the optimal combination of scaffold and signal cues that can stimulate cells and tissue in-growth into the human tooth in the cell homing process for pulp regeneration.

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Chapter 2

Implantation site impacts tissue ingrowth in cell homing study of regenerative endodontics

Abstract

Introduction: Implantation of human teeth to study cell homing into the pulp space in the rodent model has hitherto been performed at the dorsum of the animals. Ectopic tissue in the root canal specimens has been observed frequently. However, a recent cell homing study exclusively showed pulp-like tissue structure formation when immature teeth were implanted on top of rat calvaria. It was surmised, yet not tested, that the implantation site might affect tissue ingrowth in this model.

Objective: We aimed to investigate tissue ingrowth into human root canal specimens placed on top of the rat calvaria, and compared it to the ingrowth at the dorsal site.

Method: Four specimens of 3 mm-thick root sections (n = 20) from human immature third molars were implanted per rat (n = 5). Two specimens were placed at the dorsum (control) while the other two specimens were implanted at the calvaria. After 6 weeks, the specimens were investigated for histological structure, immunoreactivity to DSP and BSP, per-area percentage of tissue ingrowth, and gene expression (DSPP, COL1, NGF and VEGF). Data were statistically compared using t-test for histology and one-way ANOVA/Tukey's HSD for gene expression. The alpha-type error was set at 5%.

Results: Tooth specimens placed on top of the calvaria generally showed pulp-like tissue and odontoblast-like cells at the dentinal wall. In pre-dentin, DSP and BSP immunoreactivity

were intense underneath odontoblast-like cells. The area of tissue ingrowth was significantly (P<0.05) larger in the root canal of specimens placed at the calvaria compared to the dorsum. DSPP gene expression was also significantly higher when specimens were implanted at the calvaria (P<0.05). COL1, NGF and VEGF expression, however, did not differ significantly between the two sites.

Conclusion: Our findings suggest that the calvarial site is superior to the dorsum to study pulp regeneration in human teeth in the rat.

Keywords: cell homing, pulp regeneration, *in vivo* model

2.1 Introduction

An optimal treatment for immature permanent teeth with a necrotic, infected pulp is still elusive. In these cases, root growth is ceased and the formation of dentin is arrested after the standard endodontic treatment [1]. Poor characteristics of such teeth, i.e. their wide-open apex and large pulp cavity are not suitable for mechanical root canal debridement and normal obturation [2]. Traditional root filling materials such as MTA and gutta-percha are also inadequate to strengthen the tooth and promote its growth [2]. In addition, the biting force may cause eventual tooth loss because of hard-tissue fractures in the long term [3]. An alternative treatment has been then proposed to preserve tooth vitality and to reduce those undesirable outcomes, which has been termed “pulp regeneration”, or more accurately, “pulp revascularization” [4]. The approach has recently been established in clinics to promote pulp tissue regeneration by stem cells from the periapical region of the immature permanent tooth [5]. Instead of filling the root canal with a synthetic material, bleeding is induced for a blood clot to fill the entire canal space and act as a scaffold for regeneration. Radiographic monitoring of healing using this approach showed that the root can continue to grow, yet results are not predictable [6, 7]. There is a growing number of case reports on drawbacks and unsatisfactory results, such as insufficient bleeding, root calcification and discoloration of the tooth crown [8].

Multiple alternative approaches based on tissue engineering research have been proposed to regenerate the dental pulp. Among these, so-called cell-free approaches stand a higher chance of being transferrable to clinics [9]. A cell homing method was first introduced to the field of regenerative endodontics by the Mao group [10]. Human teeth were filled with a scaffold material combined with a mix of growth factors and chemokines. The teeth were ectopically placed at the rat dorsum. The newly formed tissue in the root canal showed an irregular pattern. Some studies using this approach even revealed abundant ectopic tissue type

formation in the pulp space such as brown and white adipose tissue [6, 11]. In addition, the embedding of tooth specimens in the dorsal area showed delayed cell migration and remnants of scaffold material [12]. In contrast, a recent study using a fibrin gel scaffold showed proper pulp structure formation after implanting the immature teeth in the rats on top of the calvaria [13]. The study showed not only the advantages of the fibrin scaffold to home the cells but it also suggested a new animal model to study cell homing for pulp regeneration. The calvarial space may provide a similar microenvironment to that of the tooth socket with various potential cell sources. It might thus be a better location for implanting teeth than the dorsal area in cell homing studies.

Therefore, we aimed to investigate the effects of the implantation site in an animal model for pulp regeneration. Pulp-like tissue formation was compared in teeth placed on top of the calvarial bone of the rat to that obtained in a control procedure at the dorsal area.

2.2 Materials and methods

2.2.1 Experimental teeth

Human immature third molars (n = 20) with open root apices supplied by the center of dental medicine, University of Zurich, were used for the current experiments. Ten teeth were taken for histology and the others were used for gene analysis. Written informed consent was obtained by all donors according to the recommendation of the Swiss Academy of Medical Science [14] . All these teeth were extracted for reasons not related to this study and stored in 0.1% Thymol at 4°C. Personnel handling the teeth applied all necessary precautions for infection control. Ethical guidelines were followed [15], and anonymisation was performed in accordance with state and federal law [16].

2.2.2 Preparation of teeth and root canals

Soft tissue remnants at outer root surface were removed using a curette. The teeth were disinfected in 5% NaOCl for 5 min in an ultrasonic bath (TEC-25, Benzer Dental AG, Zürich, Switzerland) at 60 W and 33 Hz. Roots were standardized to a length of 3 ± 1 mm by cutting the crown 1 mm above the cemento-enamel junction using a diamond-coated disc under water cooling. The pulp tissue was removed using a barbed broach (Dentsply Maillefer, Ballaigues, Switzerland). No further mechanical treatments were performed. Root canals were ultrasonically cleaned with 5 mL of a laboratory-grade 5% NaOCl solution for 5 min in an ultrasonic bath. Subsequently, 5 mL of a 17% EDTA solution (Kantonsapotheke, Zurich, Switzerland) was applied for 5 min. All root canals were finally immersed in 5 mL of a normal (0.9%) saline solution for 5 min. Teeth were kept sterile in 70% ethanol in a safety cabinet at room temperature until implantation.

2.2.3 Fibrin gel preparation and placement

A day before the implantation teeth were placed under the sterile bench to allow the evaporation of the 70% ethanol overnight. Subsequently, a 1% fibrin gel was prepared and injected into the root canals before implantation of the tooth specimens in the rats, as described previously [13]. Frozen human fibrin and thrombin dilution prepared from a Tisseel kit (Baxter, Zurich, Switzerland) were suspended in Tris-buffered saline solution at a pH of 7.4 to form a fibrin gel. The final gel was formed by mixing 8 mg/mL fibrinogen, 2.5 mmol/L Ca++, and 2 National Institutes of Health U/mL thrombin. Specimens were grouped to 4 teeth per rat with similar size of the pulp space. Two teeth were placed on top of calvarial bone while the others were implanted at the dorsum. The gel was injected from a sterile syringe into the pulp space using a 26-G cannula (Sterican, B.Braun Medical, Crissier, Switzerland). Subsequently, the specimens were stored in a humid sterile chamber at 37°C until implantation, which was performed within 2 hours.

2.2.4 Implantation

Five Sprague Dawley rats (200 - 250 g) were used for these experiments. All the following procedures were approved by the institutional ethics committee for animal research. The rats were anesthetized with ketamine and maintained with Isoflurane/oxygen during the operation. A longitudinal incision was performed using a scalpel. The blunt end of surgical scissors was then used to dissect the underlying tissue and create space for the tooth specimens. Two tooth specimens were placed subcutaneously in the rats on top of the calvaria. The other two teeth with similar size were surgically placed in the subcutaneous space at the dorsum (Fig. 1). Primary wound closure was performed using a surgical staple (3M Health Care, Rueschlikon, Switzerland).

2.2.5 Assessment of tissue ingrowth

Rats were euthanized 6 weeks after implantation in a CO_2 chamber. The tooth specimens were explanted and immediately fixed in 4% paraformaldehyde. Specimens were dehydrated and embedded in Technovit 9100 New (Heraeus Kulzer, Wehrheim, Germany) at -2°C according to the manufacturer's instruction. The resin blocks were longitudinally cut at the middle of root canal in mesiodistal plane prior to sectioning 0.5-µm-thick slides with a microtome (Leica Microsystem, Heerbrugg, Switzerland). Five subsequent sections were prepared from the center of each specimen, of which three sections were used for histological analysis and assessment of tissue ingrowth. The other two sections were kept for immunohistochemical staining. The slides were stained with Goldner's Trichrome and the images of them were taken using a slide scanner (Zeiss, Feldbach, Switzerland). Quantitative analysis was performed using freely available software (Image J; National Institutes of Health, Bethesda, MD). The areas of ingrown tissue were normalized to the total pulp space area and averaged the 3 sections per specimen, and mean values per specimens were used for the statistical comparison between groups (see below).

2.2.6 Immunohistochemistry

Immunohistochemical localizations of bone sialoprotein (BSP) and dentin sialoprotein (DSP) were observed in additional serial sections from the center of the specimens. Histologic slides were prepared as mentioned in the previous study [13] and incubated with primary antibodies (Merck Millipore, Darmstadt, Germany) against BSP (1:200) at 4°C overnight and DSP (1:100) at room temperature for 5 h. 3,3' –diaminobenzidine tetrahydrochloride (DAB) was used to detect the immunoreactivity of both markers. Slides were counterstained with haematoxylin and observed under light microscopy.

2.2.7 RNA isolation from fresh tissue

Total RNA was isolated from cells in the tooth specimens stabilized in RNAlater solution (Qiagen, Hilden, Germany). The ingrown tissue was removed from the root canal using a pin-pointed forceps, and the dentinal wall was scraped to take out the odontoblast-like cell layer using a sharp curette. Cells from both areas were processed separately according to the protocol supplied with the TRIzol reagent (Life Technology, Luzern, Switzerland). To purify RNA, the silica-membrane from RNeasy mini kit (Qiagen) was used in accordance to manufacturer's instruction. Quantity and purity of the RNA was determined using NanoDrop ND-1000 spectrophotometer (Witec AG, Littau, Switzerland) and Ethidium bromide visualizing Agarose gel, respectively.

2.2.8 Real time RT-PCR

Two-step real time RT-PCR was used for DNA quantification. 200 ng of total isolated RNA was reverse-transcribed into cDNA using a cDNA synthesis kit (Roche, Basel, Switzerland). The master mix was prepared following the manufacturer's instruction. Real-time PCR was performed using the LighCycler® 480 DNA SYBR Green I master (Roche). The rat-specific forward (F) and reverse (R) primers including DSPP, VEGF, NGF, and COL I were used in

this experiment, and GAPDH was the housekeeping gene to normalize for internal gene expression (Table 1). The 25 ul of master mix briefly consisted of 2µl of cDNA and 0.3 µM of primer. Normalized gene expression was analyzed using the $2^{-\Delta Ct}$ method and was compared statistically between groups as previously reported [17].

2.2.9 Statistics

Non-parametric method was used to compare the area of tissue ingrowth between two groups (Mann-Whitney U test), whereas parametric methods were used in comparison of gene expression regarding the normally distributed data (Shapiro-Wilk test). The difference in gene expression was thus tested with one-way analysis of variance (ANOVA) followed by Tukey's HSD for multiple comparisons. The significance level was set at 5% (P<0.05).

Gene	Primer sequences (5'-3')	Reference no.
DSPP	Forward : ACACAGGACAACCAGAATCTCA	NM_012790.2
	Reverse : CGTTGCTGTCTTTACTTCCACT	
COL I	Forward : ATCAGCCCAAACCCCAAGGAGA	NM_053304.1
	Reverse : CGCAGGAAGGTCAGCTGGATAG	
VEGF	Forward : CTACCTCCACCATGCCAAGT	NM_001287114.1
	Reverse : ACACAGGACGGCTTGAAGAT	
NGF	Forward : ACATCAAGGGCAAGGAGGTGAC	XM_006233053.2
	Reverse : TGACAAAGGTGTGAGTCGTGGTG	
GAPDH	Forward : GAAGGGCTCATGACCACAGT	NM_017008.4
	Reverse : GGATGCAGGGATGATGTTCT	

Table 1. Primer for gene analysis

2.3 Results

Histologic analysis after transplanting the tooth sections for 6 weeks revealed that all specimens were filled with connective tissue and contained a newly formed tissue (Fig. 1C). In the tooth placed on top of calvarias, more than half of root canal was occupied with a highly vascularized and innervated connective soft tissue (Fig. 1D and 1E). The regenerated tissue also showed the typical cell layers of a dental pulp (Fig 1F). At the dentinal wall, odontoblast-like cells were found in intimate contact with dentin and were positive to BSP, a marker for odontoblast function (Fig. 1G and 1H). Regenerated dentin was also observed underneath odontoblast-like cells (* in Fig. 1G) and showed DSP immunoreactivity along the dentinal wall at the interface between new dentin and old dentin (Fig. 1I). In contrast, the transplanting of tooth specimens to the dorsum showed various types of ingrown tissue (Fig. 1J) and remnants of the fibrin gel originally contained in the root canal space (Fig. 1K). The local cells were attracted into the root canal and then formed a mixture of tissue types, including fatty tissue with vacuoles (Fig. 1J) and dense collagenous tissue (Fig. 1L).

Comparison of the ingrowth related to the total pulp area showed a significant difference between two embedding sites ($P<0.05$, Fig. 2). The percent of tissue ingrowth in the tooth specimens from calvarias displayed a median of 90% (IQR = 80-94) while the tooth specimens from dorsum showed a median of 57% (IQR = 49-74). Accordingly, a measurement showed better cell migration and tissue formation at the calvarial area compared to that of the dorsum.

Gene expression of the different implantation sites was evaluated by the normalized gene expression in cells from two areas; cells contacted to the dentinal wall and from the bulk tissue. After analyzing 6 specimens from each area, DSPP gene expression levels were significantly ($P<0.05$) higher in the specimens placed on top of the calvaria compared to counterparts from the dorsum. The DSPP gene was especially up-regulated in cells from the

dentinal wall (Fig. 3). On the other hand, the expression of COL I, NGF and VEGF did not differ between the two implantation sites.

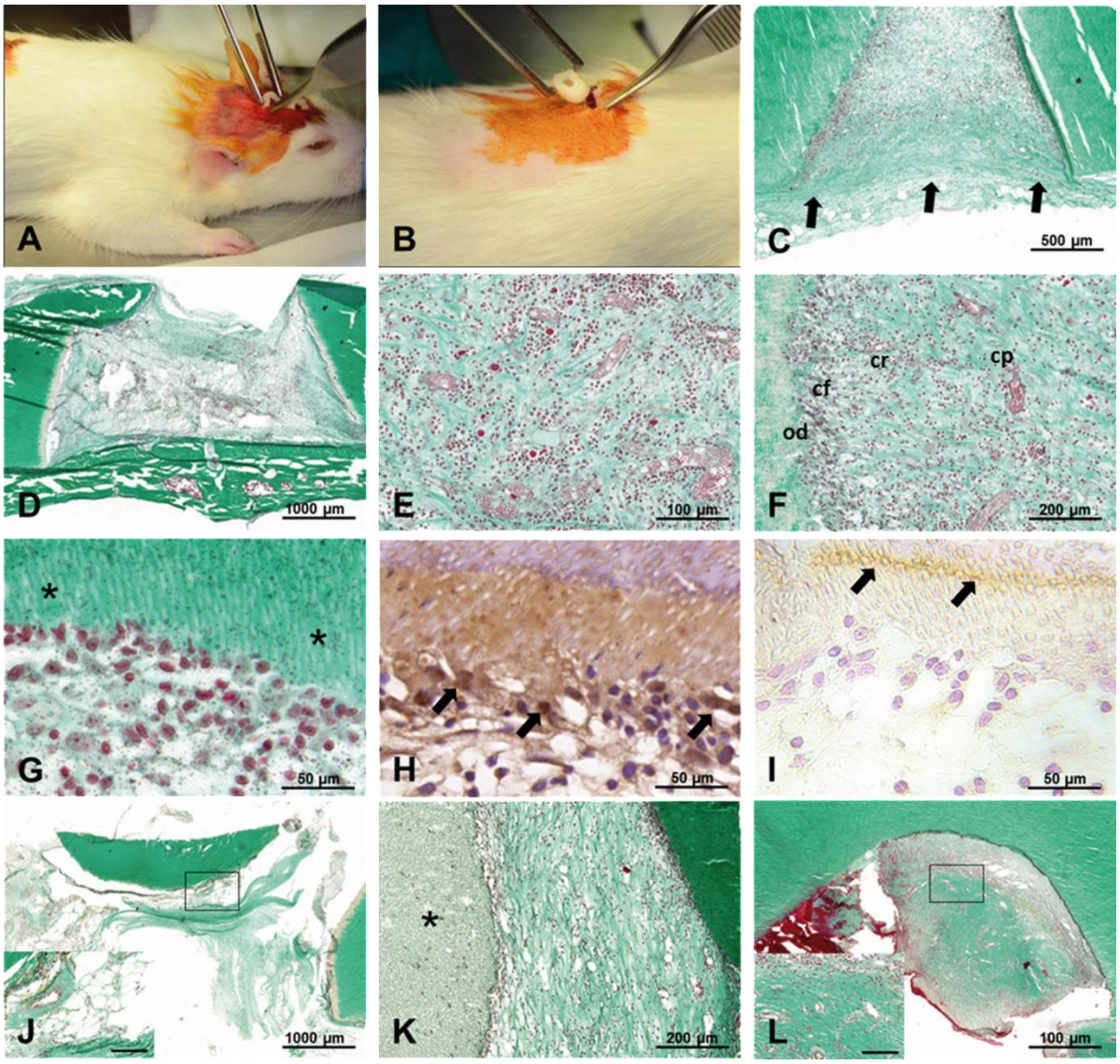

Fig. 1 Endodontically treated tooth specimens placed in subcutaneous space on top of calvaria and at dorsum of the rats. Surgical placement was performed in the same rat at calvaria (A) and at dorsum (B). 6 weeks embedding showed a fibrous layer that encapsulated the tooth specimens (arrow, C). Newly formed tissue of tooth specimens placed at the calvaria is illustrated in D-I. Overview of the regenerated tissue located in the root canal (D). Inner regenerated tissue showed revascularization and innervation (E). The tissue structure was arranged into 4 layers as normal pulp tissue (F) including central pulp (cp), cell-rich zone (cr) and cell-free zone (cf) and and apparent odontoblast cell layer (ol). Newly formed tubular

dentine (*) was observed at the dentinal wall underneath odontoblast-like cells (G). Immunohistochemical analysis revealed BSP (H) and DSP (I) in the area indicated with bold arrow. The regenerated tissue in tooth specimens placed at dorsum is presented in J-L. Low magnification image shows the irregular type of newly formed tissue (J). Adipose tissue (enlarged image in J), fibrin gel remnants (L) and dense collagenous tissue (L) were observed in the root canal. The histological slides were prepared from resin embedding block and stained with Goldner's Trichrome.

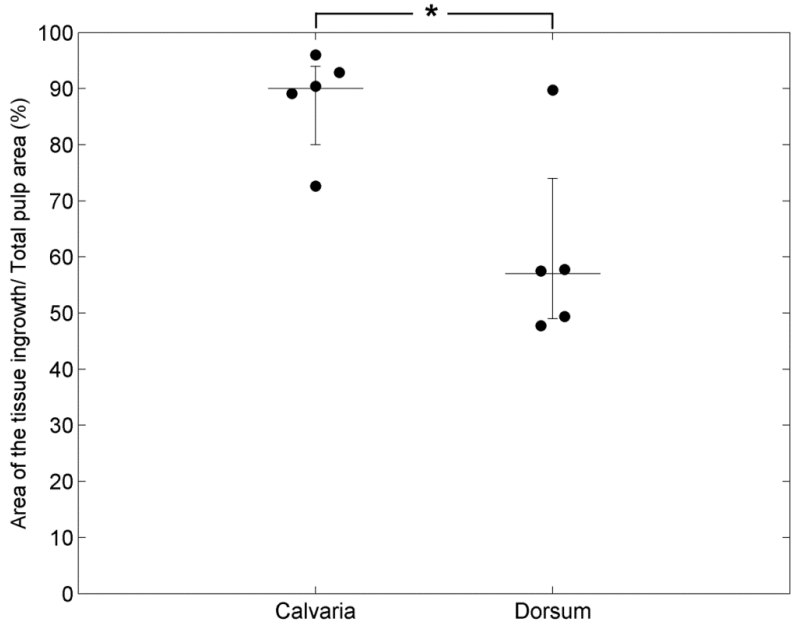

Fig. 2 Specimens placed on top of calvaria showed greater area of tissue ingrowth. 3-mm tooth sections were implanted in the subcutaneous area at the dorsum and on top of calvaria for 6 weeks. Histomorphometric analysis with image J was used to quantify the area of the tissue ingrowth and the total pulp space. Statistical analysis showed that area of tissue ingrowth in specimens implanted on the calvaria was significantly higher than in counterparts implanted at the dorsum (*, $p<0.05$). Dot plot; • indicates averaged value from each specimen, ⊥**:** Median value and Interquartile range of the group.

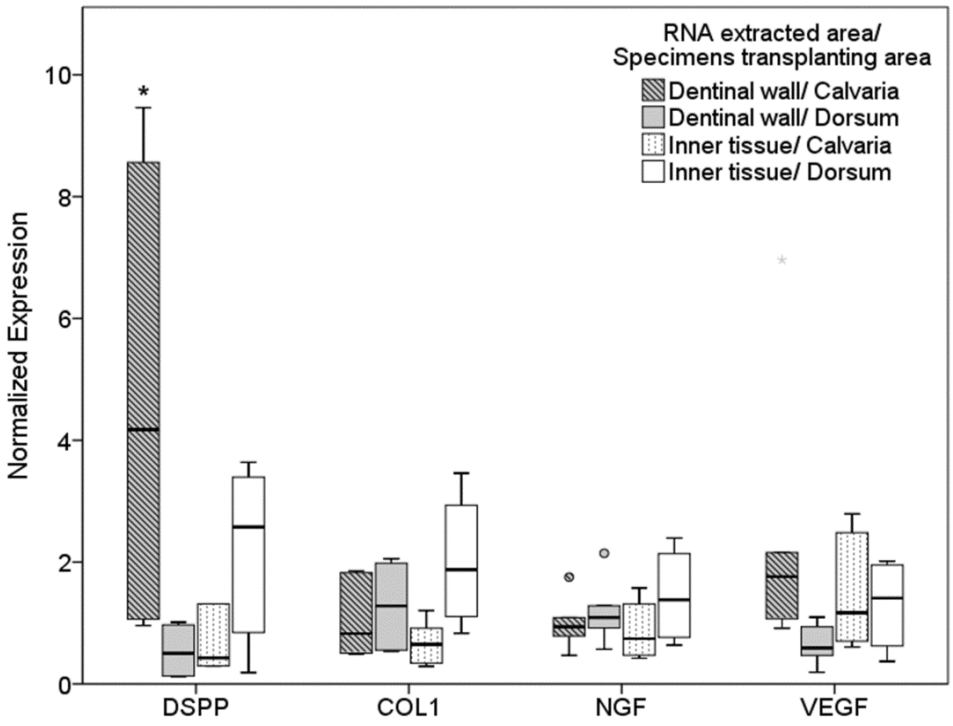

Fig. 3 DSPP gene expression was highly up-regulated at the dentinal wall of specimens implanted at the calvaria for 6 weeks. Four genes, DSPP, COL1, NGF and VEGF were analysed regarding their expression level in specimens placed at the calvaria and the dorsum. Cells at dentinal wall of the teeth at the calvaria up-regulated the DSPP gene significantly compared to cells at the dentinal wall of dorsal specimens, or cells from the inner regenerated tissue of calvarial specimens (* , $p < 0.05$).

2.4 Discussion and conclusion

In this study, we tested a novel site for implanting tooth specimens for cell homing studies in rodents. Apparently, rat calvarias offer a more suitable cell population and optimized conditions for the formation of pulp-like tissue than dorsal tissues in these animals.

Our study is limited by the fact that it remains to be shown to which extent the implanting area and the tooth preparation methods mentioned in this context are translational to clinical application. It has been argued that for pulpal regeneration, the apical papilla of immature teeth plays a key role [18]. Consequently, immune-suppressed rodents were used to be able to

implant human teeth with the cells from the apical papilla [19]. However, from a clinical standpoint, it remains unclear to which extent the apical papilla is still present in teeth with long-standing apical lesions. Furthermore, from a general health perspective, the time and money spent on pulp regeneration studies can only be justified if regenerative concepts can eventually be extrapolated to mature teeth. In mature teeth, there is no apical papilla, and pluripotent cells have to be recruited from the blood stream or the periapical area [20]. In this context, the current animal model may be more relevant than previously described simulations.

The current animal model, implanting endodontically prepared tooth specimens on top of calvarias, resulted in apparent true pulp regeneration rather than reparation. Recent reports have raised concern that a cell-free approach might yield a sequence of wound healing instead of regeneration [7, 21]. However, our results showed connective tissue revitalized with blood vessels and peripheral nerve-containing tissue, which was histologically rather similar to normal dental pulp tissue. This ingrown tissue contained odontoblast-like cells integrating into dentinal tubules, and an area of re-mineralized dentin underneath these cells. Additionally, gene analysis showed up-regulation of DSPP at the root canal wall, which is the expected location [22]. In contrast, specimens implanted at the dorsum of animals showed a different pattern of gene expression. Instead of pulp connective tissue, dense fibrous tissue or ectopic mineralized tissue was found in the root canal space. Although the dorsal space is the ideal spot in the rodent to implant a maximum amount of tooth specimens, placing the tooth in proximity to calvaria appears to yield an experimental environment that is superior to study cell homing in regenerative endodontics.

An interested finding was that in the immature permanent tooth a vital pulp-like tissue grew without implantation of stem cells. The different tissue types formed in the root canal reveal distinct cell sources between the calvarial and the dorsal area. Although tooth specimens were

similarly placed in the subcutaneous space in both areas, the supracalvarial space apparently contains cells that can form dental pulp-like tissue, which was already shown in our previous study [13]. Cells residing in the periosteum appear to be the source for this approach. These cells have been studied extensively, and their regenerative potential is used in multiple applications [23]. Periosteum-derived progenitor cells feature multi-differentiation potency and the ability to proliferate rapidly. They are the reservoir for bone regeneration and exhibit a profile similar to fibroblasts [23, 24]. It would appear that the spatial conditions of teeth in jawbone are similar to those at the calvaria. Nevertheless, further investigation of the involved cell population should be performed to better understand the current animal model.

It should be noted that the formation of pulp-like tissue in the root canal space is not only influenced by the location of implantation, but also by chemical conditioning of the canal wall. EDTA irrigation can help to promote a functional pulp by the liberation of TGF-β and other important molecular cues from the mineralized aspect of the root dentin [12, 13, 25-27]. Irregular tissue formation has been reported in pulp regeneration when mere NaOCl was used to clean in the root canal [7, 21]. NaOCl is necessary for disinfection and the dissolution of necrotic pulp tissue remnants [28, 29]; however, it has adverse effects on physical tooth properties and deproteinizes the dentin surface [25, 30, 31]. On the other hand, EDTA can preserve the vitality of stem cells, and promotes differentiation of dental pulp stem cells cultured on dentin discs [12, 25]. Obviously, the irrigation of EDTA as used in this study converts the immature tooth to be a bioactive scaffold.

We filled the canal space with fibrin gel, which acts as a scaffold for immigrating cells [32]. The selection of the scaffold in the cleaned and disinfected pulp space is also considered to be essential for the success of tissue engineering in the current context [8, 11, 33]. Fibrin gel appears to closely mimic a blood clot, without the inherent propensity of the latter to discolor the tooth. It is a material that can be modified easily to suit the application and has been

approved by Food and Drug Administration [34]. Compared to other hydrogels it shows better results in cell proliferation and differentiation [35]. The modified concentration of fibrinogen in the gel also enhances cell reactions [32]. As in the current study, we used the minimal fibrinogen concentration in an attempt to prevent cells from forming ectopic tissues in the root canal. Apparently, the fibrin in its current composition fulfills many criteria of an ideal scaffold for pulp regeneration. The translation of this gel to clinics, however, still requires modifications, such as opacity for radiographic monitoring of placement, gelation time, and other factors that might affect a convenient and safe application.

In conclusion, a proper cell source in the calvarial area, a supportive tooth preparation method, and a suitable scaffold in the pulp space appear to be a promising and relevant strategy to study cell homing during pulp regeneration. Using the current approach, more studies can now be done to further the cause of regenerative endodontics, and finally translate it into clinics.

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Chapter 3

Characterization of cells occupying the pulp space of ectopically transplanted human teeth in rodents.

Abstract

Introduction: Bleeding infiltration has demonstrated the capability to allocate mesenchymal stem cells into the empty dental pulp space. However, results on the revitalized tissue from various animal models and preliminary clinical data showed great variability in tissue formation in the root canal. A recent study by our group showed that a properly disinfected and EDTA-conditioned pulp space filled with a fibrin gel gave rise to the formation of dental pulp-like tissue as well as odontoblast-like cells integrating with dentinal tubules. Here we want to follow up on these observations.

Objective: This study focuses on the characterization of the migrated cell population and its properties related to cell homing in a fibrin gel during pulp regeneration.

Method: Human teeth were disinfected and EDTA conditioned, the pulp space filled with a fibrin gel and implanted on the calvarial bone of rats. Fibrin gel-filled silicone tubes served as controls. The specimens were implanted for three or six weeks for different analysis. Three weeks after implantation the teeth and tubes were harvested. To characterize the tissue in the teeth and the tubes, histology was performed and expression of DSP and BSP determined by immunohistochemistry. Fibrin gels from teeth and tubes were harvested and cells residing within fibrin gels were enzymatically extracted for use in flow cytometry to detect MSC

markers $CD45R^-$ and $CD105^+$. Moreover, harvested cells were cultured *in vitro* and multi-lineage differentiation potency (odonto/osteogenic and adipogenic) assessed. Six weeks after implantation, harvested cells from fibrin gel inside the teeth and tubes were used for RNA extraction to study the expressions of DSPP, COL1, NGF and VEGF by qPCR. Data were considered statistically significant when the p-value was ≤ 0.05.

Results: In tooth specimens, DSP and BSP protein were localized at the root apex and at the dentin interface but could not been detected in silicone tubes. DSPP and COL type 1 mRNA in the root canals compared to the silicone tubes were increased 3.5 and 4 fold; NGF and VEGF expression, however, was statistically similar ($p > 0.05$). The fraction of cells in the root canals negative for CD45R and positive for CD105 was 3 fold higher than in cells isolated from silicone tubes. The surface marker characteristics together with plastic adherence, cell morphology and multi-lineage differentiation potential suggested that a small fraction of the cells invaded into the pulp space had MSC characteristics.

Conclusion: Cell homing using fibrin gel appears to recruit cells, which, together with molecular cues from the EDTA-conditioned root canal wall, are able to generate pulp-like tissue.

Keywords: cell homing, pulp regeneration, immature tooth, fibrin gel, mesenchymal stem cells

3.1 Introduction

Regenerative endodontics is one of the dental disciplines where dental tissue can not only be repaired but also regenerated. The concept seems simple, since only a very small amount of pulp tissue needs to be formed [1]. The process, however, is not as trivial, since it requires complex specialized connective tissue cooperating with dentin and surviving in an environment with restricted blood flow.

An increasing number of clinical cases currently report that a functional pulp can be regenerated in immature permanent teeth most likely from stem cells originating from the dental papilla [2-5]. To that end, bleeding is induced to stimulate an influx of mesenchymal stem cells (MSCs) together with blood from lacerating the apical papilla, so that the immature tooth resumes root development [4]. This application of irreversible pulpitis of the immature tooth also leads to the formation of loose connective dental pulp like tissue and aligned odontoblast-like cells on a predentin layer [5].

Although the revascularization technique is promising, there are many reports on adverse outcomes, a formation of cementoid and/or osteoid tissue [6-8]. These unwanted outcomes likely occurred as a result of migration of stem cells from other sources into the root canal and their differentiated into ectopic cell types [7]. However, in a previous study, pulp-like tissue was formed under conditions with no stem cells present from apical papilla and without delivering exogenous stem cells [9]. That study used only fibrin hydrogel as a scaffold in combination with EDTA-released intrinsic factors from dentin to induce pulp tissue regeneration.

Here we use a newly established model for regenerative endodontics [9] and characterize the cells in terms of MSC characteristics and compare them to cells migrated into silicone tubes.

3.2 Materials and methods

3.2.1 Specimen preparation

Human immature teeth with open root apices were collected from patients at the Center of Dental Medicine, University of Zurich, with written informed consent according to the recommendation of the Swiss Academy of Medical Science [10]. All these teeth were extracted for reasons not related to this study and stored in 1% thymol at 4°C. Handling procedure followed the ethical guidelines [11] and, when relevant, the Human Research Act [12]. Teeth with multiple roots were selected and disinfected with 5% NaOCl before proceeding. The teeth were horizontally cut at the cemento-enamel juction to seperate the root sections from the crowns. Roots and sterile-silicone tubes (Faust laborbedarf AG, Schaffhausen, Switzerland) were cut into pieces of 6 mm in length (n=10 for each group) and, in diameter, 2.5±0.6mm for the roots and 3mm for the tubes. All specimens were irrigated with 5% NaOCl, followed by 17% EDTA as previously described [9]. Intracanal and intralumen were finally flushed with 0.9% normal saline solution to remove remnants of EDTA. The coronal access of the root canals and one opened side of the silicon tubes were subsequently closed with a glass ionomer cement (Ketac Molar®, 3M-ESPE, Seefeld, Germany), and all specimens were kept sterile in 70% ethanol.

3.2.2 Fibrin gels

1% Fibrin gels were prepared by mixing 8 mg/ml fibrinogen, 2.5 mmol/L Ca++, and 2 National Institutes of Health U/mL thrombin (Baxter, Zurich, Switzerland), all in Tris-buffered saline solution at a pH of 7.4. 50 µl of the fibrin gel was injected into dried root canals and silicone tubes by using a 1 ml syringe and a 26-gauge canula (Sterican, B.Braun Medical, Crissier, Switzerland). The complete gelation took 30 min under standard cell/tissue culture conditions before specimens were implanted in the rats.

3.2.3 *In vivo* embedding

All protocols for animal experiments were approved by the Institutional Ethics Committee for Animal Research. 200 - 250 g female Sprague Dawley rats were used in an *in vivo* cell homing model described previously. In brief, the tooth and tube specimens were placed subcutaneously on top of calvarial bone of the rats, which were anesthetized using ketamine followed by Isoflurane/oxygen. A longitudinal incision was made prior dissection the underlying tissue by blunt end scissors to create space, where the teeth and tubes were placed horizontally with the opening facing opposite sides. Primary wound closure was performed using a surgical staple (3M Health Care, Rueschlikon, Switzerland). Euthanization was done 3 weeks or 6 weeks later using a carbon dioxide chamber.

3.2.4 Histological analysis

Specimens were immediately fixed in 4% paraformaldehyde and followed by serial dehydration. In this study, Technovit 9100 New (Heraeus Kulzer, Wehrheim, Germany) was used to embed the teeth at -2°C according to the manufacturer's instruction. The resin blocks were longitudinally cut at the middle of the root canal in the mesiodistal plane prior to sectioning 0.5 µm thick slides using a microtome (Leica Microsystem, Heerbrugg, Switzerland). All histological images were obtained using a light microscope.

3.2.5 Immunohistochemical staining

Immunohistochemical localizations of bone sialoprotein (BSP) and dentin sialoprotein (DSP) were observed in additional serial sections from the center of the specimens. Histologic slides were prepared as previously described [9] and incubated with primary antibodies (Merck Millipore, Darmstadt, Germany) against BSP (1:300) and DSP (1:300) at 4°C overnight. Prior to peroxidase-conjugated streptavidin incubation, biotin-conjugated goat-anti rabbit and rabbit-anti mouse secondary antibody were used for a second staining step at room

temperature for 30 minutes. Immunoreactivity of both markers were detected by 3,3' diaminobenzidine tetrahydrochloride (DAB). Slides were counterstained with haematoxylin and observed under light microscopy.

3.2.6 Gene expression

Collected specimens at 6 weeks were immediately immersed in RNALater (Qiagen, Hilden, Germany) at 4°C overnight before storing at -80°C until use. Total RNA was isolated using TRIzol® (Life Technology, Luzern, Switzerland) from cells within the fibrin gel. A purification step was performed using a silica membrane column (Qiagen). The complementary DNA (cDNA) was transcribed from 200 ng RNA using a synthesis kit (Roche, Basel, Switzerland). For the gene expression analysis, real-time RT-PCR was performed using the LighCycler® 480 DNA SYBR Green I master (Roche) and 25 µl of master mix containing 2 µl of cDNA, 12.5 µl of SYBR Green, 0.3 µM of primer and RNase free water. The rat-specific forward and reverse primers for DSPP, VEGF, NGF, and COL type I were used in this experiment, and normalized with GAPDH (Table 1). Quantitative PCR (qPCR) was performed in the subsequent three steps: 2 min at 50°C, 15 min at 95°C, followed by 55 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Target gene expression was compared between cells from root canals and silicone tubes. A two-fold change was a minimal criterion to consider the outcome as an up-regulated expression.

Gene	Primer sequences (5'-3')	Reference no.
DSPP	Forward : ACACAGGACAACCAGAATCTCA	NM_012790.2
	Reverse : CGTTGCTGTCTTTACTTCCACT	
COL I	Forward : ATCAGCCCAAACCCCAAGGAGA	NM_053304.1
	Reverse : CGCAGGAAGGTCAGCTGGATAG	
VEGF	Forward : CTACCTCCACCATGCCAAGT	NM_001287114.1
	Reverse : ACACAGGACGGCTTGAAGAT	
NGF	Forward : ACATCAAGGGCAAGGAGGTGAC	XM_006233053.2
	Reverse : TGACAAAGGTGTGAGTCGTGGTG	
GAPDH	Forward : GAAGGGCTCATGACCACAGT	NM_017008.4
	Reverse : GGATGCAGGGATGATGTTCT	

Table 1. Primer for gene analysis

3.2.7 Isolation of cells and flow cytometry

Fibrin gels from teeth and tubes were separated from the surrounding tissue and cells residing in the fibrin gel were dispersed enzymatically into a single cell suspension. The ingrown tissue was incubated with 1 mg/ml collagenase/dispase (Sigma-Aldrich, Darmstadt, Germany) in a water bath at 37°C for 4 hours. The digestion of the cell suspension was stopped by dilution with phosphate buffer saline (PBS) and then passed through a 100-µm Nylon cell strainer (Sysmex, Horgen, Switzerland). Cells were washed twice with PBS and counted using a Neubauer chamber. $1x10^5$ cells from each group were first blocked with 3% bovine serum albumin (BSA) in 1 mM EDTA/PBS for 30 min. The single-cell suspensions (10^6 cells/ml) were incubated with rat primary antibody specific for CD105 (Merk & Cie,

Schaffhausen, Switzerland) for 45 min and followed by a master mix of PE/Cy7-conjugated secondary antibody and APC-conjugated antirat-CD45R (Biolegend, San Diego, Switzerland) for 45 min. As a negative control, incubation buffer was added. All staining steps were done at 4 ˚C under light protection and washing was performed with 1 mM EDTA/PBS between each step. The cells were finally fixed with 10% formalin for 10 min at room temperature and repeatedly washed with 1 mM EDTA/PBS prior to flow cytometry analysis (Sysmex-Partec, Görlitz, Germany). FlowJo v.10 was used to analyze the data. A preliminary gate was set up regarding the multi-size bead kit (Sysmex) and compensation was done using AbC Anti-Rat bead kit (Thermofisher, Steinhausen, Switzerland). The gating on CD45R-negative cells was used to determine a positive population of CD105.

3.2.8 Cell expansion

Isolated cells were seeded and maintained in a growth medium containing α-minimum essential medium (α-MEM; Thermo Fisher scientific) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher scientific) and 1% (v/v) penicillin/streptomycin (P/S; Thermo Fisher scientific). At 80% confluence, cells were harvested by treating with trypsin-EDTA (Thermo Fisher scientific) and then were reseeded in a T-75 flask for expansion. Cell passages 3 and 4 from both tubes and teeth were used in the analysis while human mesenchymal stem cells (hMSCs) from bone marrow, obtained by Ehrbar's laboratory (University Hospital Zurich), were cultured in parallel as a control.

3.2.9 *In vitro* differentiation

Cells harvested from fibrin gels were further characterized by their multi-lineage differentiation potential. hMSCs and tooth/tube-isolated cells were seeded in a 24-well plate in the growth medium before specific induction. Three days after cells reached 70% confluence, a differentiation medium was added into each well. To induce cell differentiation

to odonto/osteoblast and adipocyte, cells were incubated with a specific medium for 21 days [13]. Briefly, the odonto/osteogenic medium was the growth medium supplemented with 100 nM dexamethasone, 10 mM b-glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich), and two different media for adipogenic induction contained 10 mg/ml insulin, 10 mM dexamethasone, 100 mM indomethacin, and 500 mM 3-isobutyl-1-methyl xanthine, during adipogenic induction, or 10 mg/ml insulin, during adipogenic maintenance (Sigma-Aldrich). The formation of odonto/osteoblast was assessed by ALP activity as previously described [14]. Calcium deposition was evaluated with Alizarin red staining (Sigma-Aldrich) at pH 4.2. Finally, the accumulation of lipid vacuoles was determined by oil Red O staining (Sigma-Aldrich) and the absorbance measured at 500 nm.

3.2.10 Statistical analysis

Following a normality test (Shapiro-Wilk), data was compared the difference of means in SPSS 22.0 using student's *t*-test for two samples and analysis of variance for multiple samples. Tukey's test was subsequently used to find a difference between groups. A significant difference was considered at the 95% confidence interval.

3.3 Results

3.3.1 Histological analysis

The histology of the cross sections of teeth and tubes revealed cell ingrowth into both cavities. Tissue formation, however, only occurred in the pulp space of human teeth. In the silicone tube, a high number of uniform cells could be found in the fibrin matrix (Fig 1A). Those cells showed no integration or interference with the silicone wall. Cells within the teeth appeared more heterogeneous (Fig 1B). A fraction of the cells appeared to attach to the dentinal wall while centered cells either remained single cells and resembled the cells in the

silicone tube or changed their morphology and appeared fibroblast-like. No mineralized tissue could be identified at three weeks.

3.3.2 Expression of marker genes

The expression of marker genes from teeth and silicone tubes was evaluated at 6 weeks after implantation. COL type I and DSPP, were found to be upregulated 3.5 and 4.0 fold respectively in root canals compared to silicone tubes (Fig 2). VEGF and NGF mRNA content, however, did not differ. The increase in DSPP expression could be confirmed by immunoreactivity of DSP and BSP, where for both proteins positive areas could be found within the root canals but not in silicone tubes (Fig 3, A-D). Intense staining for both proteins could be observed at the dentinal wall and at the root apex (Fig 3, G-H), while no staining could be detected at all in the silicone tube (Fig 2, E-F).

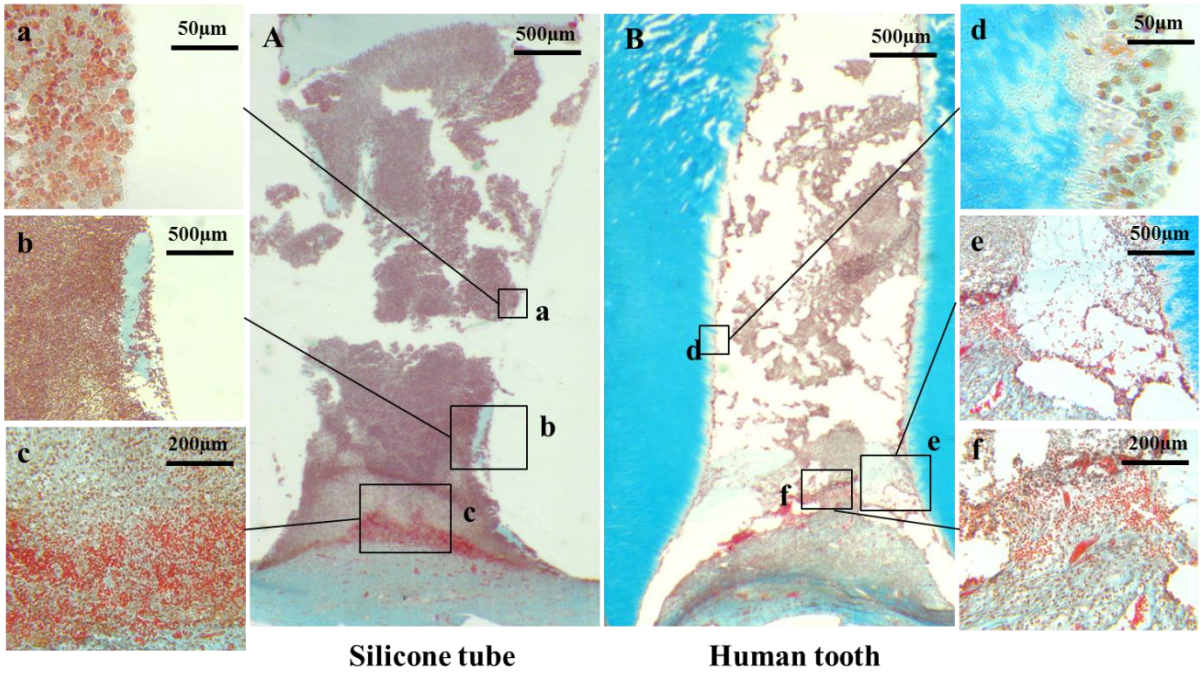

Fig. 1 Histologic images 3 weeks post embedding silicone tubes and human teeth in rat. Goldner's tricrome staining was used to show ingrowth within fibrin gel of both specimen types. A: Cells located commonly inside lumens of the silicone tubes. (a) Due to space limited by surrounding wall, migrated cells were densely packed at the border. (b) A high number of cells was found within fibrin gel where (c) a fibrous cap separated it from external tissue. B: In contrast, fewer cells appeared to be recruited into the human root canal. (d) Three weeks after transplantation, the mobilized cells were able to integrate well with the dentinal wall. (e) In the teeth, more cells are located at the periphery interacting with the dentinal wall. (f) Connective tissue supplemented with blood vessels was established at the apical third

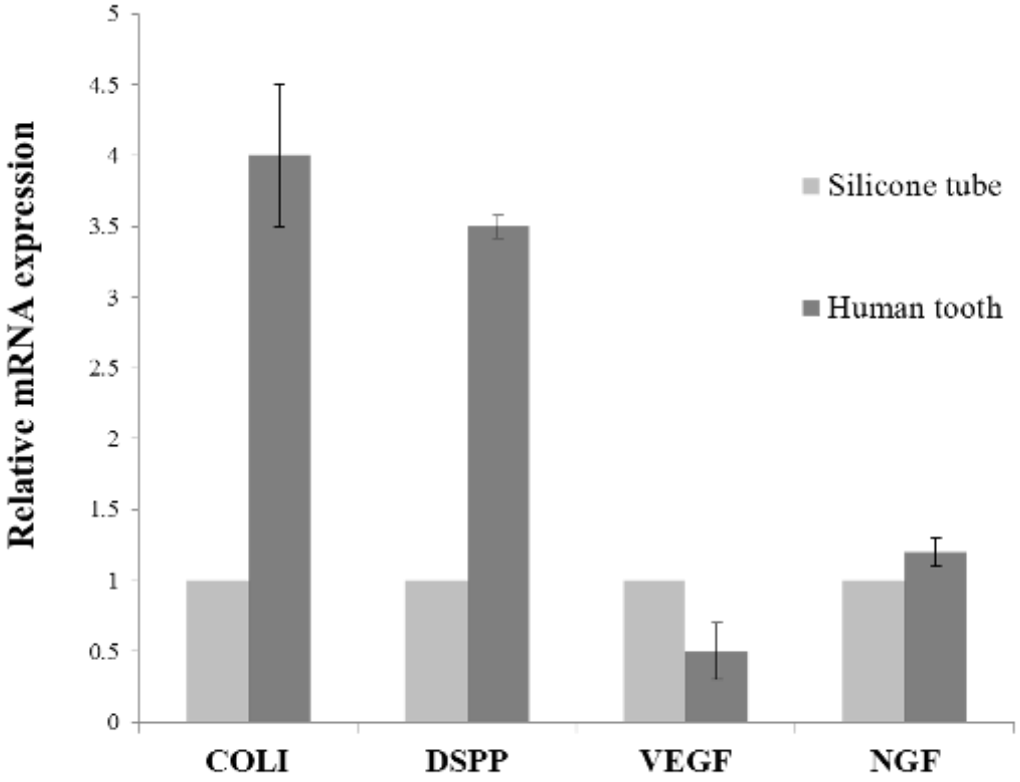

Fig. 2 Effect of dentin matrix on the expression of marker genes at 6 weeks. DSPP, COL type I, VEGF and NGF mRNA content was compared between cells harvested from human root canals and from silicone tubes (GAPDH control).

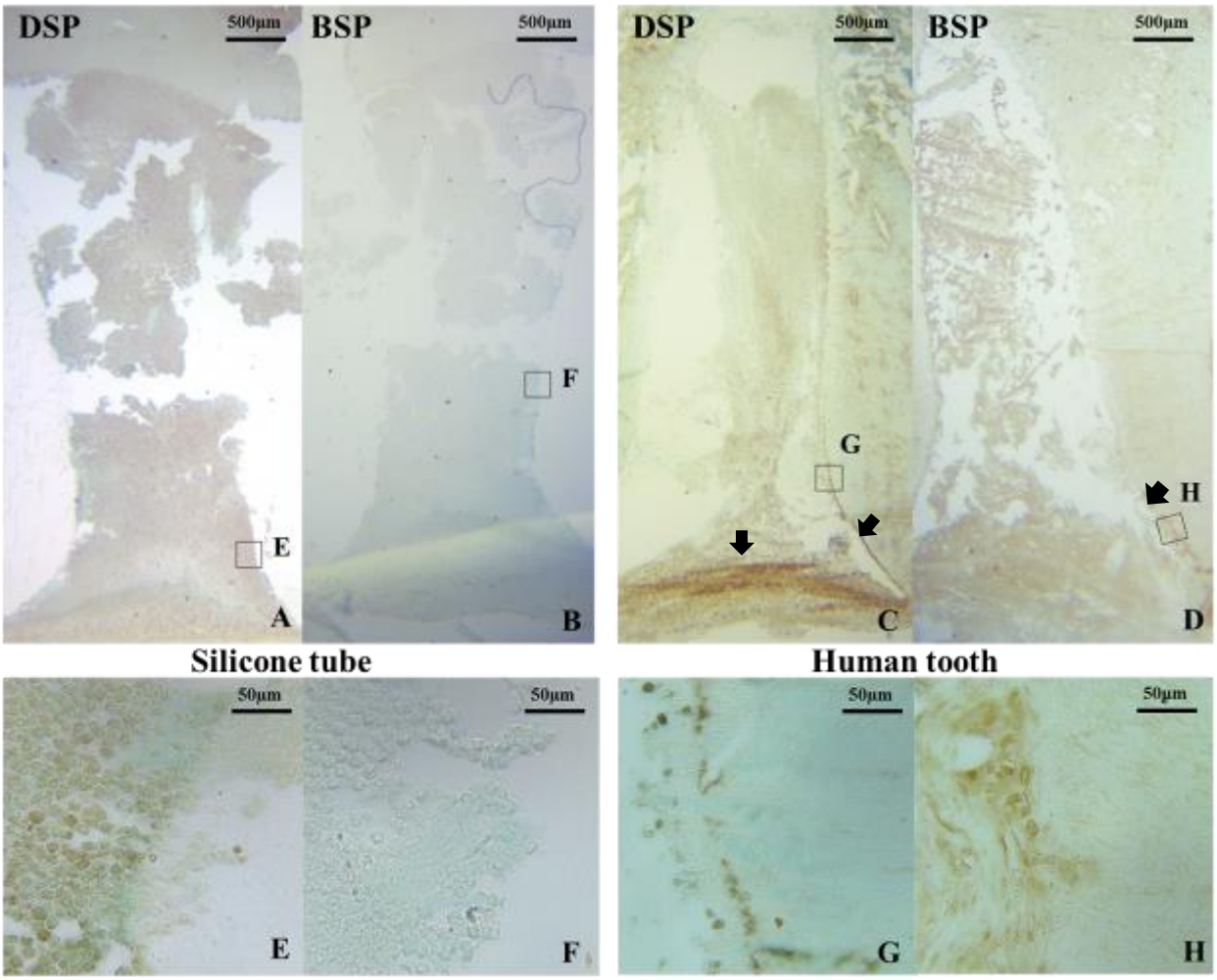

Fig. 3 Immunoreaction to dentin sialoprotein (DSP) and bone sialoprotein (BSP) antibody within the tube lumen and the root canal space. (A-B) Three weeks following transplantation to the rat, no immuno-positive area could be detected in the silicone tube. (E-F) Higher magnification at the periphery revealed no cells positive to these antibodies. (C) In the root canal, migrated cells were intensively immunoreactive to the DSP antibody at the dentinal wall and the root apex (arrow). (D) BSP staining was also revealed at the dentin interface. (G-H) Zoom in on images of the immuno-positive areas showed only cells contacting dentin.

3.3.3 Flow cytometry

To further characterize the cells found after 3 weeks in the pulp space and the lumen of silicone tubes, flow cytometry was performed (Fig 4). The plot of size (FSC) versus granularity (SSC) showed that overall morphology of the cells from tubes and teeth was similar. A serial gating was performed as shown in Fig 4A. The number of cells harvested from the silicone tubes (18±7 $x10^3$ cells/ml) was significantly higher compared to human root canals (8±2 $x10^3$ cells/ml) (Fig 4B). The cells displaying negative for CD45R and positive for CD105 were 5.2±2.2% of the cell population in the root canals, which was significantly higher than the 2.0±1.2% found in the tubes (Fig 4C). Taking into account the overall number of the cells, the absolute number of CD45R negative and CD105 positive cells was similar in teeth and tubes.

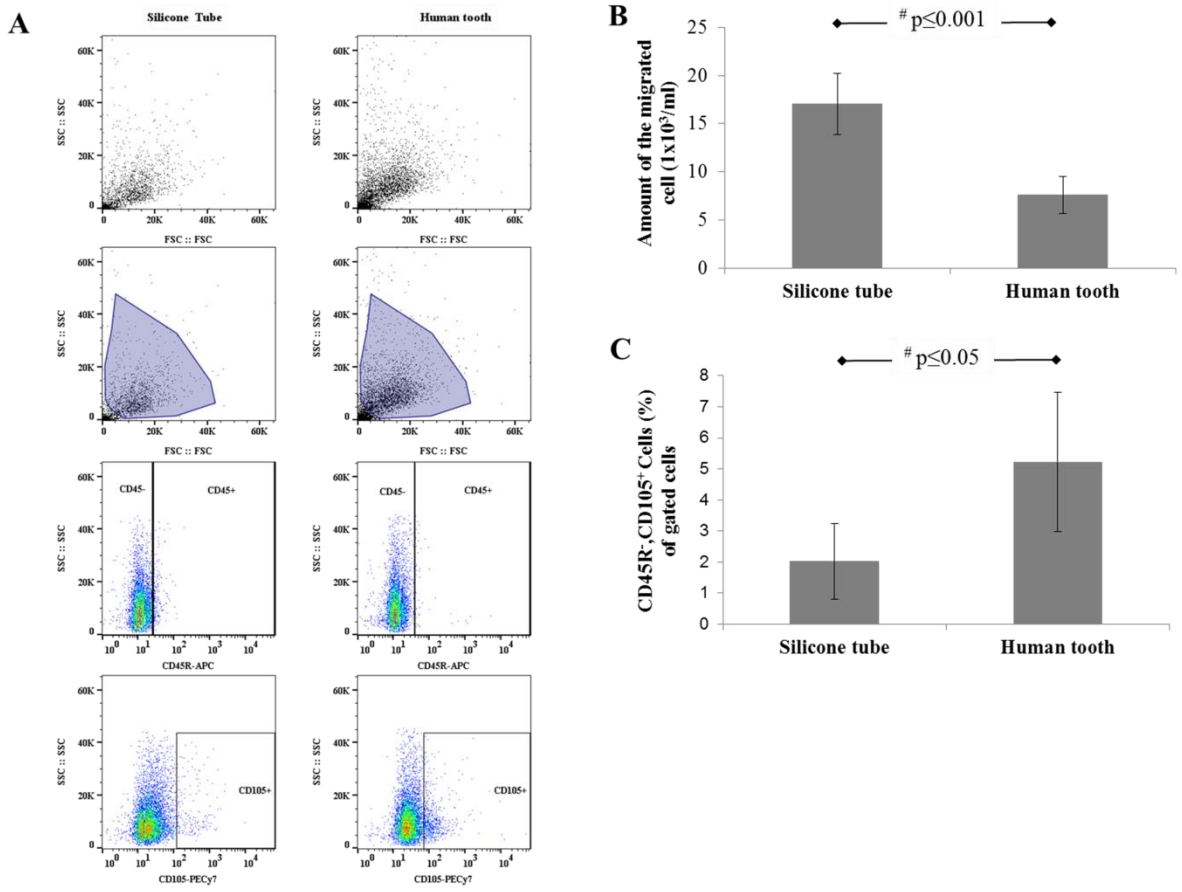

Fig. 4 Characterization of the isolated cells from silicone tubes compared to human teeth. Specimens filled with fibrin gel were implanted on top of calvaria of rats for 3 weeks. (A) Preliminary cell selection was performed dependent on size and granularity. Cells that negatively expressed APC-conjugated CD45R were included for the evaluation of CD105-PECy7. (B) The amount of isolated cells was counted using a Neubauer counting chamber. Cell number within the tubes was significantly higher than in the root canals ($p \leq 0.001$). (C) The number of cells negative for CD45R and positive for CD105 was significantly higher in preparations from teeth compared to preparations from the silicone tube ($p \leq 0.05$). Bar charts represent mean values and standard deviation of data.

3.3.4 MSC-like characteristics of expanded cells

Cells harvested from fibrin gels in human teeth or silicone tubes were subsequently expanded *in vitro*. Cells from both specimens were able to attach to a plastic cell culture flask and could expand under standard culture conditions as used for hMSCs (Fig 5A). Various cell

morphologies could be identified within both cell sources. However, cells from human teeth were more fibroblastic-like and resembled hMSCs more than the cells harvested from the silicone tubes (Fig 5). .

To observe cell plasticity potential, cells isolated from human pulp space and the silicone tube were compared to hMSCs. Under odonto/osteogenic induction at 21 days, cells harvested from teeth and cells harvested from tubes showed calcium nodule formation similar to hMSCs (Fig 6). As a second marker for odonto/osteogenesis, ALP activity, was determined. The ALP activity increased in stimulated hMSCs as well as cells extracted from root canal and silicone lumen compared to unstimulated hMSCs. Likewise, adipogenic differentiation potency was similar between hMSCs and tooth/tube-isolated cells (Fig 7). Their absorbance at 500 nm was significantly higher compared to the unstimulated hMSCs. However, the adipogenic potency and the ALP activity between cells extracted from human tooth did not differ from tube-isolated cells. Therefore, at 3 weeks, a small fraction of cells in the teeth and in the tube appears to have multi-lineage properties like hMSCs towards odonto/osteogenesis and adipogenesis.

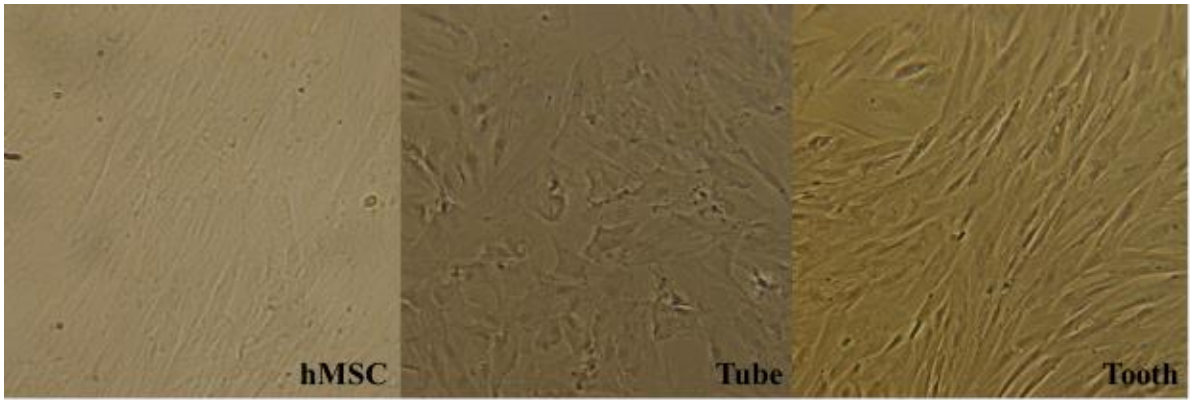

Fig. 5 Cell morphology following *in vitro* expansion. Isolated cells from *in vivo* embedding in human teeth and silicone tubes were cultured in conditions used for human mesenchymal stem cells (hMSCs). A mix of cells that was enzymatically extracted from fibrin gel within the teeth principally showed fibroblast-like characteristics similar to hMSCs. However, tube-isolated cells generally revealed more diverse morphologies

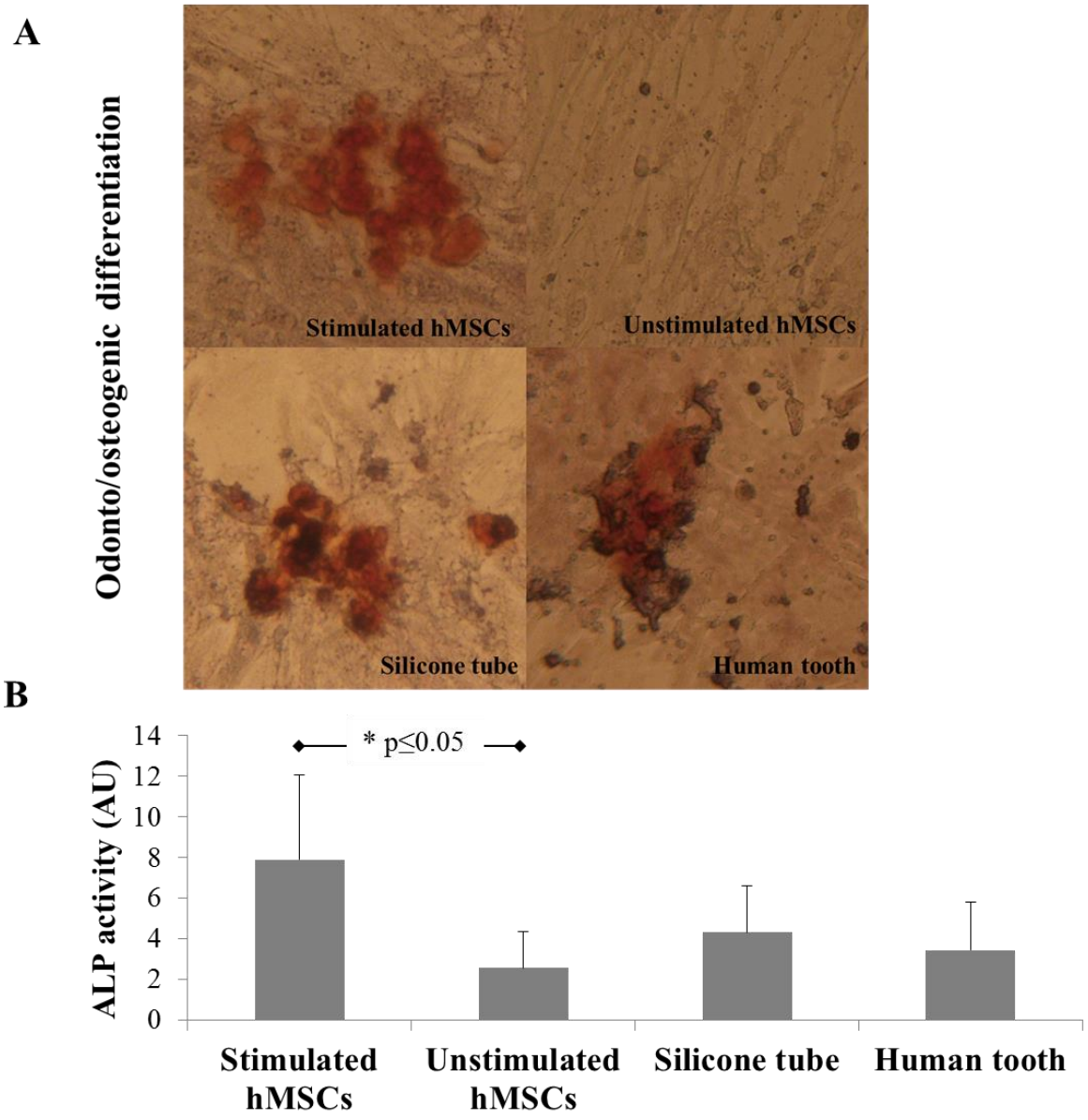

Fig. 6 Odonto/osteogenic differentiation potency of cells enzymatically extracted from human tooth and silicone tube compared to human mesenchymal stem cells (hMSCs). hMSCs and cells harvested from specimens produced calcium nodules after 21 days (alizarin red staining) and showed statistical difference in ALP activity between stimulated and unstimulated hMSCs. Bar chart represents mean value and standard deviation.

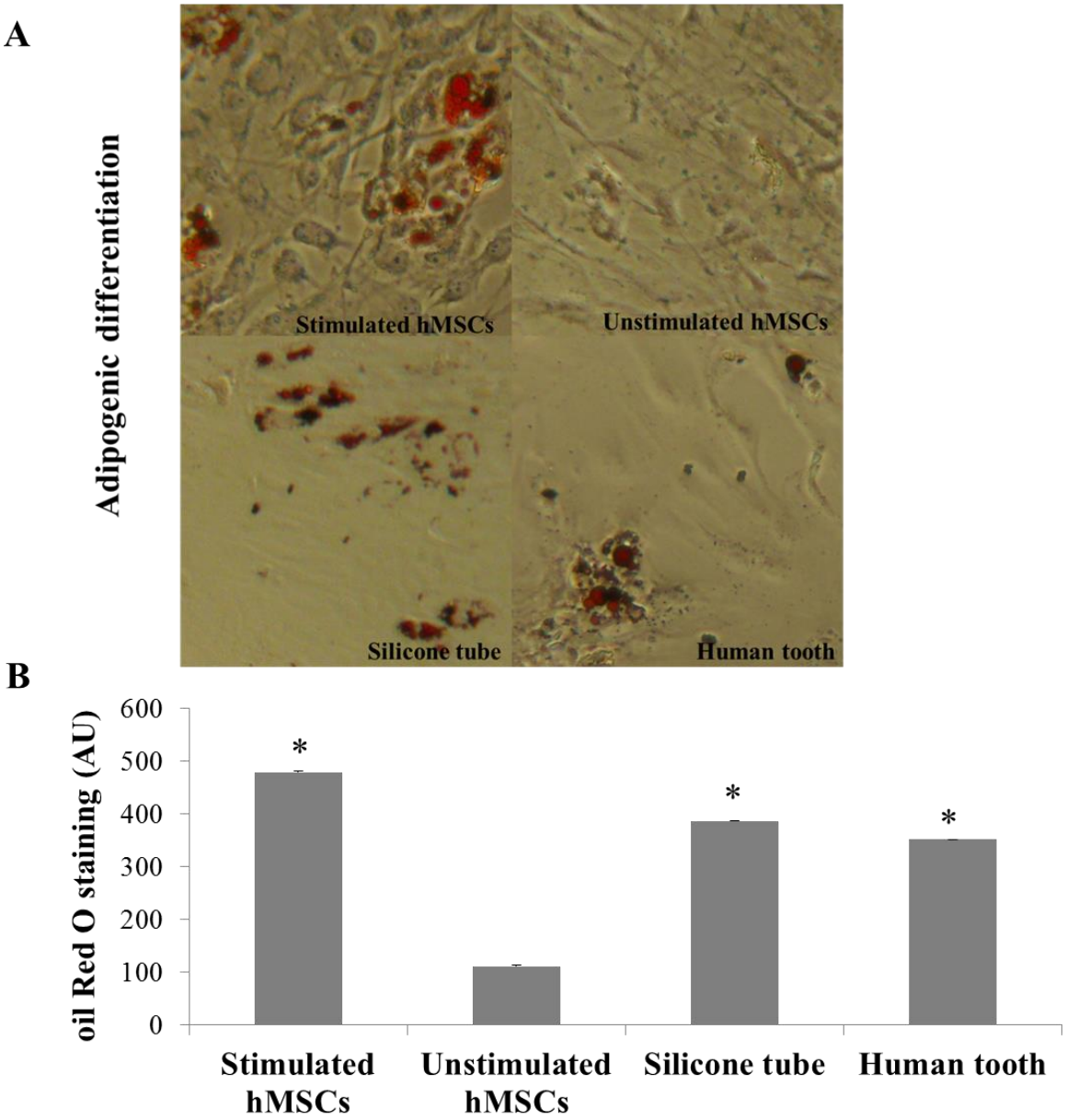

Fig. 7 Adipogenic induction of cells isolated from human teeth and silicone tubes compared to human mesenchymal stem cells (hMSCs). All cells were stimulated under the same conditions and with the same medium for 21 days. Lipid vacuoles stained by oil Red O were found in stimulated hMSCs and tooth/tube-isolated cells but not in unstimulated hMSCs. The absorbance at 500 nm of the unstimulated hMSCs was also lower compared to the other groups (∗: $p<0.05$). Bar chart represents mean value and standard deviation.

3.4 Discussion and conclusion

The presence of an apical papilla as a source for stem cells to repopulate the pulp has been seen as standard prerequisite to achieve a successful outcome with the pulp revascularization technique [15]. Results obtained with our newly developed *in vivo* model lacking an apical papilla as stem cell source suggests that even in the absence of the apical papilla a properly conditioned tooth filled with a fibrin gel is sufficient for pulp regeneration [9]. This proposes that stem cells from sources other than the apical papilla could also repopulate and regenerate the pulp. To further characterize the invaded cells in fibrin gels, we compared the cells harvested from human pulp space with cells harvested from the lumen of silicone tubes.

The use of a fibrin gel to mimic intra canal blood clotting and the intrinsic growth factors embedded in dentin showed to facilitate host endogenous cells to migrate and home in the pulp space. Although fibrin gel was previously used for bone tissue engineering, primarily to treat cranial defects [16], fibrin gels in the pulp space predominantly yielded the formation of pulp-like soft connective tissue within the root canal, occasionally with osteodentin at the dentinal wall, but mostly with tubular-like dentin structure [9]. Various cell types were found at 3 weeks post implantation in both the silicone tubes and intra root canal, but differentiation to pulp cells as manifested by DSPP expression and the presence of DSP in close proximity to odontoblast-like cells in near the dentinal wall was only found in the pulp space. In this context, fibrin gel together with the intrinsic growth factors, which were made accessible after the root canal was irrigated with EDTA, induced invading cells to differentiate towards odontoblast-like cells [17, 18]. The importance of the dentin surface and or factors released from dentin for differentiation towards odontoblasts is further underlined by an 3-4 times increased expression of DSPP and COL type I in cells harvested from the pulp space compared to cells harvested from silicone tubes. These genes are known to be expressed by odontoblasts during odontogenesis [19, 20]. The differentiation could not be detected in the

silicone tube where the growth signals are missing, although they were prepared in a similar way as the teeth.

Corresponding to those findings are areas of BSP and DSP immunoreactivity at the dentinal wall where cells adhered to the activated dentin surfaces and were able to elongate their processes into the dentinal tubules. In addition, the BSP and DSP immunoreactivity were not only present but also located at the appropriate location, both at the dentinal wall and at the opened root apex. The immunoreactivity of DSP was more intense than that for BSP, and it was suggested that this profile mimicked reparative dentin formation [21], which is different from a profile of ectopic hard tissue produced by subcutaneously placed pulp cells [22]. A direct comparison of DSP and BSP content, however, is not possible since the detection is based on different antibodies with different affinities to their antigens.

In terms of sheer numbers, we found more cells residing in the silicone tubes, although it is known that biological signals from dentin promote cell migration *in vitro* [17]. The reason for the increased number of cells in the silicone tubes could derive from the fact that cells in the pulp space might already interact strongly with the dentinal wall and cannot be recovered at all [23]. From the silicone tube, however, cells can be easily retrieved since the adhesion of cells to this material is minimal [24]. The other issue could be that teeth, in contrast to silicone tubes, are biological materials, which are more compatible, and the increased number of cells in silicone tubes could derive from a foreign body reaction [25, 26]. Another aspect that could not be taken into account is the volume of the empty space, which in the case of the tube is larger than in the case of the tooth. Although the access-opening diameter and the length were similar, the shape of the inner volumetric space differed between the tooth, and the silicone specimens, as the tube’s lumen is a straight column, whereas the immature root canal is a reversely tapered cylinder. Thus, more cells were able to accommodate within the tube than within the intra root canal.

Despite the fact that the cell number was higher in the silicone tube, the number of cells negative for CD 45 and positive for CD105 was significantly higher in the fibrin gels from teeth. Their absolute number, however, was similar. Comparable to cells observed under the current conditions, in the revascularization technique currently advocated in clinical dentistry, some infiltrating cells are of the MSC phenotype [27, 28]. Here, the cleaning with 5% NaOCl, followed by 17% EDTA, which exposes and released growth signals from dentin in addition to removing the smear layer [29-31], could be the reason that $CD45^-$ and $CD105^+$ cells enter preferentially the pulp space rather than the lumen of the silicone tubes. According to the chemical dentin preparation used in the current study, TGF-β1 is a possible signaling molecule for the CD105 expressing cells, as are bFGF and BMP2, which all promote chemotaxis for specific cell types and drive cell differentiation towards odontoblast-like cells [17, 31-33]. When MSC-like cells are exposed to these factors liberated from the dentin surface, they might lose their stem cell properties and differentiate towards odontoblast-like cells.

To further characterize the cells entrapped in the pulp space and the silicone tubes, we expanded them and subjected them to osteogenic and adipogenic conditions. In comparison to hMSCs, their potential to differentiate into the osteogenic/odontogenic lineage determined by ALP activity and mineral deposition was reduced to about 50%, but they were nonetheless still present. This reduction by 50% could also be at least partially due to the difference between odontoblastic and osteogenic differentiation, since hMSCs are derived from bone marrow but harvested cells are from cells migrating into the fibrin gel and already encountering signals from the dentinal wall [30, 34]. The difference in adipogenic potential between hMSCs and the tooth/tube-isolated cells, as determined by oil red O staining, was even smaller. Therefore, in accordance to minimal criteria for multipotent mesenchymal stem cells, including plastic adherence and differentiation to odonto/osteoblasts and adipocytes

[35, 36], such cells can be found in both the pulp space of immature teeth and the lumen of silicone tubes implanted on the calvarial bone of rats after three weeks.

The findings of this study do not disclose the origin of the immigrated MSCs. Various stem cell niches reside in the subcutaneous space and at the calvaria of the rat [37]. All of them might be a possible cellular source and the MSCs, mentioned here, could be either a single type or of mixed origin. Therefore, MSCs might not be the solely cells type contributing to pulp regeneration [38]. A functional apical papilla, however, is not a prerequisite for pulp regeneration, which opens the possibility to perform pulp regeneration procedures also with mature teeth [27].

In conclusion, MSCs are recruited into the treated human root canal and, into silicone tubes. Specialized cell differentiation and the maturation of dedicated connective tissue, however, occur only in fibrin gel filled teeth. These findings indicate that properly surface-modified dentin can turn fibrin gels into an active scaffold for cell homing in regenerative endodontics. From this perspective, the clinical absence of apical papilla may not be a major concern, but rather it is the clinical process that is most important.

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Chapter 4

Stem cell factor: a promising chemokine for acceleration of pulp regeneration.

Abstract

Introduction: Cell homing during regenerative endodontics has shown encouraging results. However, it would still be desirable to make the process more predictable and also faster. SCF is known for its potency to guide stem cell migration and to induce repair and regeneration in a variety of organs. Fibrin gels in combination with SCF, therefore, might enhance pulp regeneration.

Objective: This study aimed to test SCF action in cell migration, cell proliferation and cell differentiation first *in vitro* and then *in vivo* using a cell homing animal model.

Method: Human mesenchymal stem cells (hMSCs) were exposed to SCF at various concentrations. Cell proliferation and differentiation towards odonto/osteoblasts was assessed by WST-1 assay and alkaline phosphatase activity, respectively. For the migration, 3D-chemotaxis slides were used together with time-lapse microscopy. For the *in vivo* experiment, fibrin gels were prepared either with or without 15µg/ml SCF in bilateral human immature premolars (n=5 for each group). Two corresponding teeth, one with SCF and one without were placed on top of rat calvaria for 6 and 12 weeks. All tooth specimens from this experiment were either analyzed histologically and the percentage of tissue ingrowth determined or the cells extracted from the pulp space and the expression of DMP1, DSPP,

COL1, NGF and VEGF assessed by qPCR. Differences were considered significant at the 95% confidence level.

Results: In the presence of SCF, we saw an increase in hMSCs directional migration, proliferation and odonto/osteogenic differentiation. *In vivo,* SCF increased the extent of tissue ingrowth at 6 weeks but not at 12 weeks. At 12 weeks the formed tissue appeared more mature in samples with SCF and odontoblast-like cells featured elongated cytoplasmic processes reaching deeper into dentinal tubules. In terms of gene transcription, DMP1, COL1 and VEGF were the significantly upregulated genes while the other genes (DSPP, and NGF) were not affected.

Conclusion: Our results suggest that SCF can accelerate cell homing in human immature teeth and the maturation of odontoblast-like cells in the pulp/dentin complex.

Keywords: cell homing, pulp regeneration, stem cell factor, immature permanent tooth

Chapter 4 Stem cell factor

4.1 Introduction

Revascularization technique applied to immature permanent teeth showed the potential that a functional pulp tissue can regenerate and continue tooth development [1, 2]. In average, it takes one to two years after intra canal blood clot formation to see significant radiographic changes of the treated teeth, including an increased root length and thickness [3, 4]. Some teeth, however, broke prior the manifestation of the tooth maturation, or took longer periods of time to accomplish mineralized tissue formation [1, 5, 6].

In regenerative endodontics, cell homing appears as a promising strategy [7, 8]. The concept depends on a scaffold, normally a cellular matrix, and growth factors or cytokines to promote host cells migration into the pulp space and finally regeneration of tissue by the host cells [7]. Various scaffold materials, such as collagen, synthetic-polypeptide hydrogels and fibrin gels, are able to promote odontoblast-like cell formation and/or dental pulp-like tissue production [9-11]. Growth factors as bone-morphogenic protein (BMP), nerve growth factor (NGF) and granulocyte colony-stimulating factor (G-CSF) have also shown to facilitate tissue growth up from the apical all the way to the coronal aspects of the root canal [8, 12]. Without these growth factors, the intrinsic signals embedded in dentin activated by root canal irrigants such as ethylenediaminetetraacitic acid (EDTA) can also produce an apparently functional pulp [10, 13]. As a result, the application of the cell homing concept appears efficient to induce repair/regenerative processes within the pulp space. Thus, an acceleration of the procedure would appear advantageous.

The first step in a cell homing approach is the attraction of cells to the diseased site. Stem cell factor (SCF) recently has been shown to be a powerful chemokine. It is the polypeptide ligand that binds to c-kit receptor (or CD 117), and has chemotactic properties to recruit progenitor cells [14]. At the site of injury, SCF was also found abundantly prior to repair processes [15]. For tissue engineering, SCF applications initiated the repair of diverse tissues

such as hearts [16, 17], liver [18] and nerves [19]. Furthermore, SCF showed to regulate protein secretion needed during proliferation, chondrogenesis and extracellular matrix formation of MSCs [20]. SCF and its receptor were also found in differentiating dental pulp cells (DP) and in the sub-odontoblastic layer of Höhl [21]. In pulp tissue engineering, a previous application of SCF on a collagen sponge either with or without DP showed an increased expression of genes required for collagen remodeling, migration and proliferation [21]. However, the application of SCF in a cell homing approach to form a fully functional pulp has not been reported yet.

The present study, therefore, used SCF to improve the cell homing concept by an acceleration of the regeneration process. *In vitro* experiments were set up to test the effect of SCF on human mesenchymal stem cells (hMSCs) and *in vivo* experiments were performed using a cell homing model in rats [10] to reveal the effect of SCF on tissue ingrowth and odontoblast maturation.

4.2 Materials and methods

4.2.1 Cell migration

Non-coated µ-Slide Chemotaxis ^{3D} assays (Ibidi, Munich, Germany) were used to study the effect of SCF on the directionality of hMSCs migration. The fully characterized hMSCs were obtained from Ehrbar's Laboratory (University Hospital Zurich). hMSCs were plated in plastic tissue culture dishes and grown in minimal essential medium (α-MEM; Thermo Fisher scientific, Steinhausen, Switzerland) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher scientific), 1% penicillin/streptomycin (P/S; Thermo Fisher scientific) and 5ng/ml FGF (Immunotool). To passage hMSCs, trypsin/EDTA (Thermo Fisher scientific) was used each time as previously description [22]. hMSCs were used from passage 4 to 6. $1x10^6$cells per ml were loaded into a middle chamber of the slide following a user guide. Pure

α-MEM was used to wash away non-adherent cells after four hours and to starve the cells four hours prior the beginning of assay. One reservoir of the cell chamber was filled with control medium (α-MEM) and the other filled with the same medium supplemented with different concentrations of SCF. To observe cell migration, the μ-slide was mounted on the stage of an inverted microscope at 37 ˚C in 5% CO_2 condition. Video microscopy for the time-lapse experiment was set up to use a 5x objective lens with phase-contrast mode and each frame was recoded every 10 min for 24 hours. Thirty cells randomly selected were tracked manually using MTrackJ in combination with the Image J plug-in. The tragedy plots and the Rayleigh test were generated by this software [23].

4.2.2 Cell proliferation

Equal number ($1x10^4$ cells per ml) of the hMSCs from passage 4-6 was seeded in 96-well plates 24 hours before changing the medium. The growth medium was prepared with α-MEM supplemented with 10% FBS and 1% P/S as a negative control. A medium containing 5ng/ml FGF additionally was used as a positive control. Growth media were supplemented with different concentrations of SCF. Measurements were done in triplicate. After 48 hours, 10% WST-1 (Roche, Basel, Switzerland) was added to each well for monitoring cell proliferation spectrophotometrically at 630 nm.

4.2.3 Odonto/osteogenic differentiation

To test the effect of SCF on odonto/osteogenic differentiation of hMSCs, passage 4-6, were seeded in 96-well plate in the growth medium. Three days after the cells reached 70% confluence, an osteogenic differentiation medium was supplied as previously reported [22]. All measurements were performed in triplicate. The growth medium and the standard differentiation medium were used as a negative and positive control, respectively. Alkaline phosphatase (ALP) activity was assessed quantitatively with a modified assay as described

earlier [24] and normalized to total cellular protein concentrations using Coomassie Brilliant Blue staining (Sigma-Aldrich). The active cells also stained with ALP staining solution, including a mixture of 0.1 mg/mL napthol AS-MX phosphate and 0.6 mg/mL Fast Blue BB salt from (Sigma-Aldrich).

4.2.4 Specimen preparation

Bilateral immature premolars with open root apices were supplied by the orthodontic department of the Center of Dental Medicine, University of Zurich. They were obtained from patients aged between 9-15 years under written informed consent according to the recommendation of the Swiss Academy of Medical Science [25]. All these teeth were extracted for reasons not related to this study and stored in pairs at -20°C. The procedure fulfilled the local ethical guidelines [26], and was in agreement with state and federal laws [27]. Teeth were cleaned and proceeded as reported earlier [10]. Briefly, they were disinfected with 5%NaOCl and accessed from the crown prior irrigating in the root canal using 5% NaOCl and followed by 17% EDTA (Kantonsapotheke, Zurich, Switzerland). Finally, 0.9% normal saline solution was used to free the teeth from remaining EDTA. The coronal access was subsequently closed with KetacMolar® (3M-ESPE, Seefeld, Germany) and all tooth specimens were kept sterile in 70% ethanol.

4.2.5 Fibrin gels and SCF

1% Fibrin gels were prepared by mixing 8 mg/mL fibrinogen, 2.5 mmol/L Ca++, and 2 National Institutes of Health U/mL thrombin (Baxter, Zurich, Switzerland), all in Tris-buffered saline solution at a pH of 7.4. Rat recombinant SCF at concentration of 15 µg/ml (immunotool, Friesoythe, Germany) was added into thrombin solution prior the mixing step. 50 µl of fibrin gel was injected into dried root canal using a 1-ml syringe equipped with a 26-gauge cannula (Sterican, B.Braun, Melsungen, Germany). Teeth were filled either fibrin gel

alone or fibrin gel combined with SCF, and the gel was allowed to form for 30 min in standard culture condition before embedding in rats.

4.2.6 *In vivo* embedding

All experimental procedures with animals were approved by the local authorities (Kantonales Veterinäramt, Zurich). Adult female Sprague Dawley rats weighing 200 - 250 g were used for these experiments. The prepared teeth with or without SCF were placed on top of the calvaria of rats as previously described [10]. The paired teeth were horizontally placed in the same animal for 6 or 12 weeks and their opened apexes turned to the opposite site (left and right).

4.2.7 Histological evaluation

Tooth specimens were collected following carbon dioxide euthanasia of the rats and were immediately fixed in 4% paraformaldehyde prior a serial dehydration with ethanol. In this study, we used Technovit 9100 New (Heraeus Kulzer, Wehrheim, Germany) to embed all specimens according to the manufacturer's instruction. The resin blocks were longitudinally cut at the middle of the root canal in mesiodistal plane before sectioning 0.5-µm-thick slides with a microtome (Leica Microsystem, Heerbrugg, Switzerland).

4.2.8 Histomorphometric assessment

Histological images were prepared as previously described [10]. Three subsequent sections were made from the center of each specimen and used for histological analysis. The slides were stained with Goldner's Trichrome and the images of them were taken using a slide scanner (Zeiss, Feldbach, Switzerland). Quantitative analysis was performed using a measurement module from Panoramic Viewer (3D-Histech, Budapest, Hungary). The areas of ingrown tissue were normalized to the total pulp space area and averaged the 3 sections per specimen, and mean values per specimens were used for the statistical comparison between groups. For the evaluation of the cytoplasmic process, three representative areas from each

tooth were randomly selected, and the extent of the process of ten odontoblast-like cells from dentin interface was measured.

4.2.9 Gene expression

Cells within the bulk ingrowth and cells attached to the dentinal wall were collected from teeth that had been embedded in the rat for 12 weeks. RNA was extracted using TRIzol® (Life Technology, Luzern, Switzerland) from RNALater (Qiagen, Hilden, Germany) preserved specimens. Total RNA was measured following the clean-up protocol using a silica membrane column (Qiagen). Complementary DNA (cDNA) was transcribed from 200 ng RNA using a synthesis kit (Roche, Basel, Switzerland). For the quantitative expression, real-time RT-PCR was performed using the LighCycler® 480 DNA SYBR Green I master (Roche) and 25 µl of master mix consisted of 2 µl of cDNA, 12.5 µl of SYBR Green, 0.3 µM of primer and RNase-free water. The rat-specific forward (F) and reverse (R) primers including DSPP, VEGF, NGF, and COL type I were used in this experiment, and GAPDH was the housekeeping gene for internal gene normalization (Table 1). Quantitative PCR (qPCR) was done in three steps as following run: 2 min at 50°C, 15 min at 95°C, followed by 55 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C. Normalized gene expression was analyzed using the $2^{-\Delta Ct}$ method and was compared statistically between groups.

Gene	Primer sequences (5'-3')	Reference no.
DMP 1	Forward : GTGACCAGACAGGGAATGGG	NM_203493
	Reverse : AGAAACGACAACAAAGCGGC	
DSPP	Forward : ACACAGGACAACCAGAATCTCA	NM_012790.2
	Reverse : CGTTGCTGTCTTTACTTCCACT	
COL I	Forward : ATCAGCCCAAACCCCAAGGAGA	NM_053304.1
	Reverse : CGCAGGAAGGTCAGCTGGATAG	
VEGF	Forward : CTACCTCCACCATGCCAAGT	NM_001287114.1
	Reverse : ACACAGGACGGCTTGAAGAT	
NGF	Forward : ACATCAAGGGCAAGGAGGTGAC	XM_006233053.2
	Reverse : TGACAAAGGTGTGAGTCGTGGTG	
GAPDH	Forward : GAAGGGCTCATGACCACAGT	NM_017008.4
	Reverse : GGATGCAGGGATGATGTTCT	

Table 1. Primers for gene analysis

4.2.10 Statistical analysis

Following a normal distributing evaluation, *in vitro* result was statistically compared by one-way analysis of variance (ANOVA) followed by Fischer's Least Significant Difference for pairwise tests. For the *in vivo* data, the difference between two samples was considered by Mann-Whitney test. All statistical tests were used SPSS 22.0 and a significant difference was considered at the 5% level. For the cell migration assay, the data was analyzed with Rayleigh test, which determined uniformity in circular distribution of cell endpoints and the p-value below 0.05 implied an unequaled movement of cells.

4.3 Results

4.3.1 *In vitro* effects of SCF on hMSCs

To examine whether SCF can direct hMSCs migration, we performed experiments in chemotaxis micro-slide chambers. Tracking the movement of cells exposed on one side to 2.5µg/ml and 12.5µg/ml of SCF, the hMSCs preferentially moved towards the SCF containing reservoirs (Fig 1A). To this end, a gradient formed by SCF from a peak concentration of 2.5µg/ml was sufficient to direct hMSCs toward the source of SCF.

Next, we examined the effect of SCF on hMSCs proliferation and odonto/osteogenic differentiation. At a concentration of 100 ng/ml SCF, hMSCs proliferation was significantly faster than in controls, i.e. the same medium lacking SCF, and the cell number increased by an average of 130% (Fig 1 B). This was higher than the hMSCs number in the medium containing 5ng/ml FGF. Below 100 ng/ml SCF no significant increase in proliferation could be observed. In terms of differentiation, 1000 ng/ml SCF increased ALP activity (26±5 unit) significantly in an osteogenic medium (Fig 1C). The SCF at high concentrations (2500 and 5000 ng/ml), however, showed a very low level of response. These results were confirmed by ALP staining (Fig. 1D). In essence, concentrations of SCF from 100 ng/ml increased proliferation of hMSCs and differentiation was only affected at very high concentrations of 1000 ng/ml SCF.

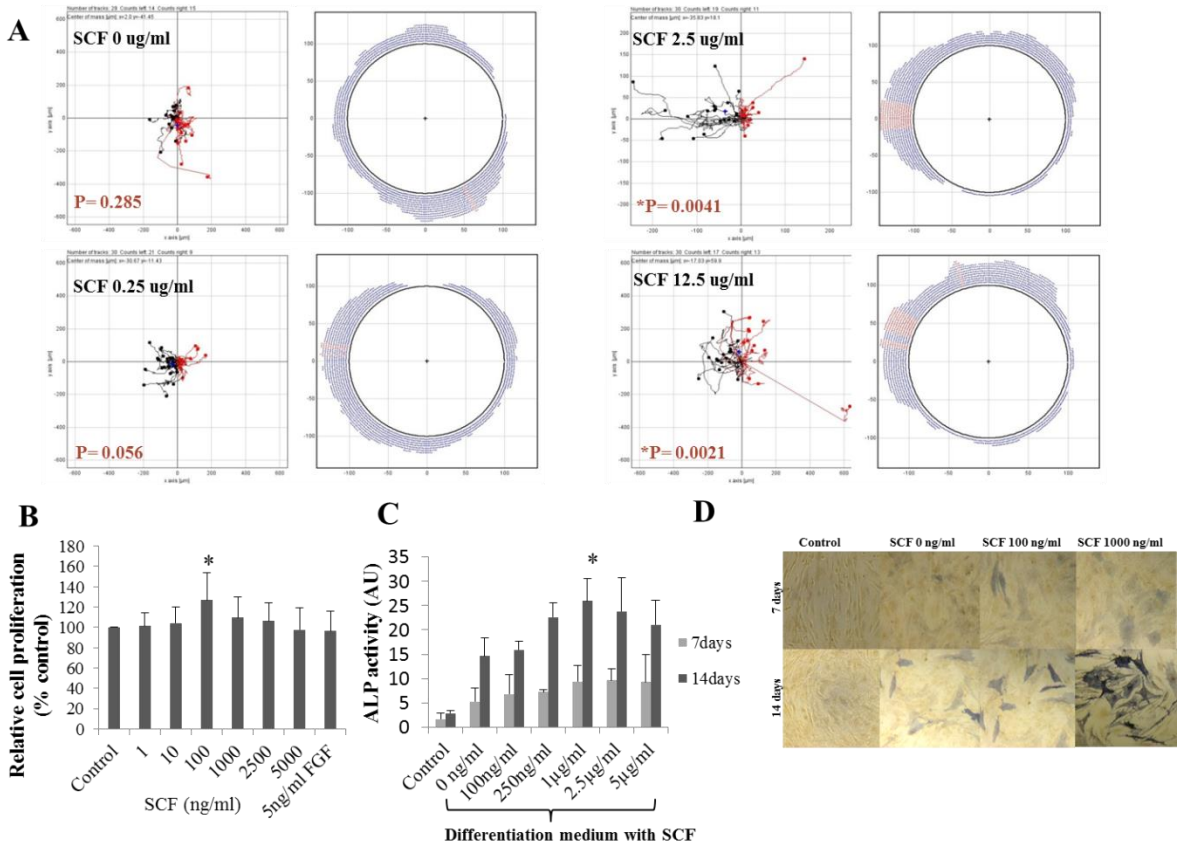

Fig. 1 In vitro effects of SCF on mesenchymal stem cells (MSCs) migration, proliferation and odonto/osteogenic differentiation A) MSCs migrated towards an SCF gradient. All plot charts depict migration over a 24 hour period from the center of mass (the average of the x and y coordinates). The red color indicates cell migration towards the medium alone, and black color indicates cell migration towards the SCF-doped medium. Rayleight test (p-values) was significant when 2.5 and 12.5 µg/ml SCF were loaded in one side of the reservoirs. **B)** WST-1 assay was used to determine hMSC proliferation dependent of SCF concentration and FGF. The relative cell proliferation was referred to the control which contained normal fetal bovine serum supplemented medium. 100 ng/ml SCF showed a significant increase in cell proliferation. **C and D)** ALP activity and staining showed the odonto/osteogenic activity of hMSCs under SCF stimulation. Growth medium was used as a control and varying SCF concentrations were applied to the osteogenic medium. 1 µg/ml SCF significantly enhanced ALP activity of hMSCs at 14 days compared to the osteogenic medium without SCF (0 ng/ml). These representative images of ALP staining confirmed its activity. * $p \leq 0.05$

4.3.2 Cell homing acceleration by SCF *in vivo*

To assess the effect of SCF on tissue ingrowth into immature teeth placed on top of the calvarial bone, we analyzed teeth implanted for 6 and 12 weeks histologically. At 6 weeks for teeth filled with fibrin gel plus 15 µg/ml SCF, the percentage of tissue ingrowth was (21± 5%) higher compared to (6±2%) with fibrin gel alone (Fig 2). At 12 weeks both numbers had increased. In the presence of SCF, tissue ingrowth was 47±15%. In teeth without SCF, tissue matured at 38± 13% of the pulp space.

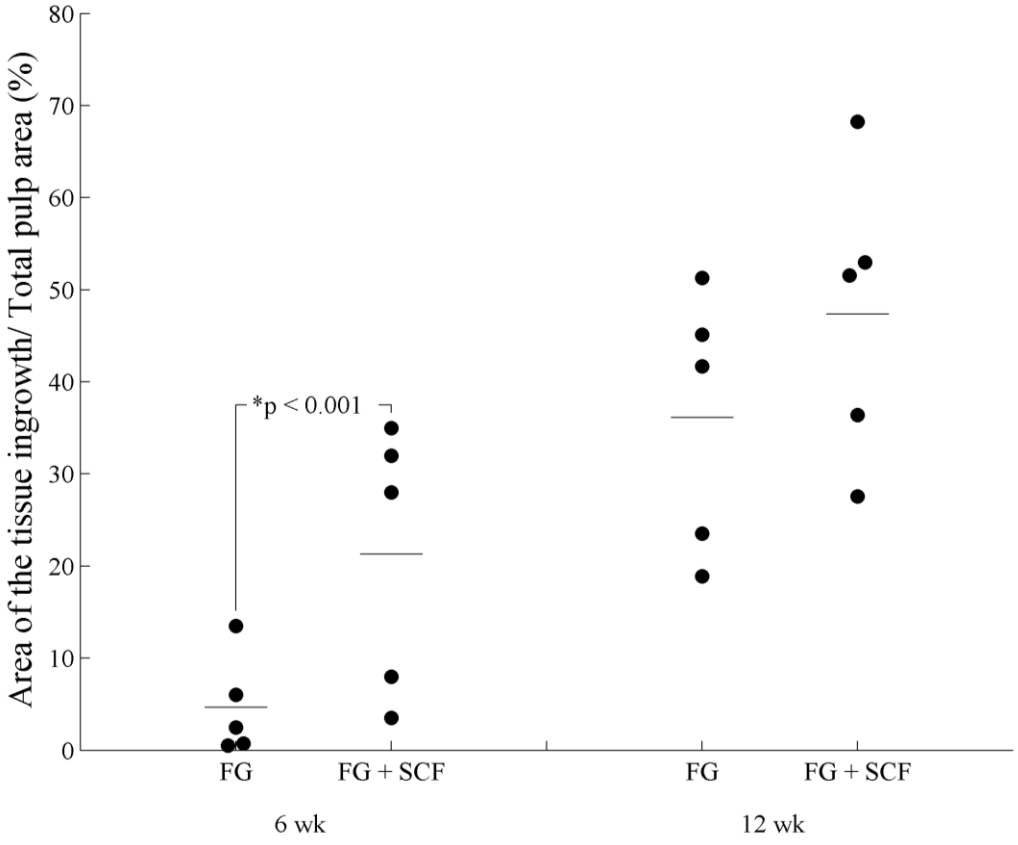

Fig. 2 The effect of SCF on tissue ingrowth. Bilateral human premolars filled with fibrin gel alone (FG) or in combination with 15µg/ml SCF (FG+SCF) were implanted on top of the rat calvaria for 6 and 12 weeks. The percentage of the pulp space where tissue had grown in was determined based on the middle sections from the histologies. The average value of each group is indicated by a horizontal bar. While both intervals showed a tendency towards greater tissue formation with the SCF-loaded fibrin gel compared to the pure fibrin gel, the mean values were statistically significant (*p≤0.05) only at 6 weeks, but not at 12 weeks.

Histology of bilateral human premolars harvested after 6 weeks revealed that tissue ingrowth was accelerated by the application of SCF, and the morphology of this tissue was also affected (Fig. 3). In absence of SCF, ingrown tissue was found at the apical third of the root canal only and appeared to be in an early stage of development, since areas of accumulated red blood cells were still visible. Immature mineral matrix was also below half of the old-predentin thickness. Newly-formed hard tissue could not be detected at the dentinal wall or the apical opening, regardless of whether SCF was applied or not. The stage of tissue maturation, however, was progressed in presence of SCF-doped fibrin gels. First, the area of tissue ingrowth reached up to the middle third of the root canal and second, early blood vessel formation together with red blood cells could be appreciated. Third, the immature mineral matrix reached along the entire predentin layer.

At 12 weeks the effect of SCF on tissue ingrowth was not significant anymore (Fig. 2, 4) but the formed tissue in specimens containing SCF was more mature. Without SCF, the in-grown tissue matured up to the middle third of the root canal. It contained highly developed blood vessels together with odontoblast-like cells at the dentinal wall, whereas only a connective tissue developed to close the opened apex. A similar pattern could be seen in the presence of SCF. However, the tissue appeared more diversified. First, hard tissue had developed at the apical opening and second, revascularization appeared to be improved. Third, the odontoblast-like cells extended their processes further down the dental tubules. As shown in figure 5, in presence of SCF the odontoblastic process reached 87 ± 6 µm into the dentin, while in absence of SCF the corresponding value was 26 ± 3 µm.

On the level of DSPP and NGF expression, no difference was seen between fibrin gels/SCF and fibrin gel only samples. DMP1 and COL1 mRNA content in SCF loaded fibrin gel significantly upregulated in cells attached to the dentinal wall (Fig. 6). In the tissue ingrowth, VEGF was slightly but it was the solely gene upregulated by SCF.

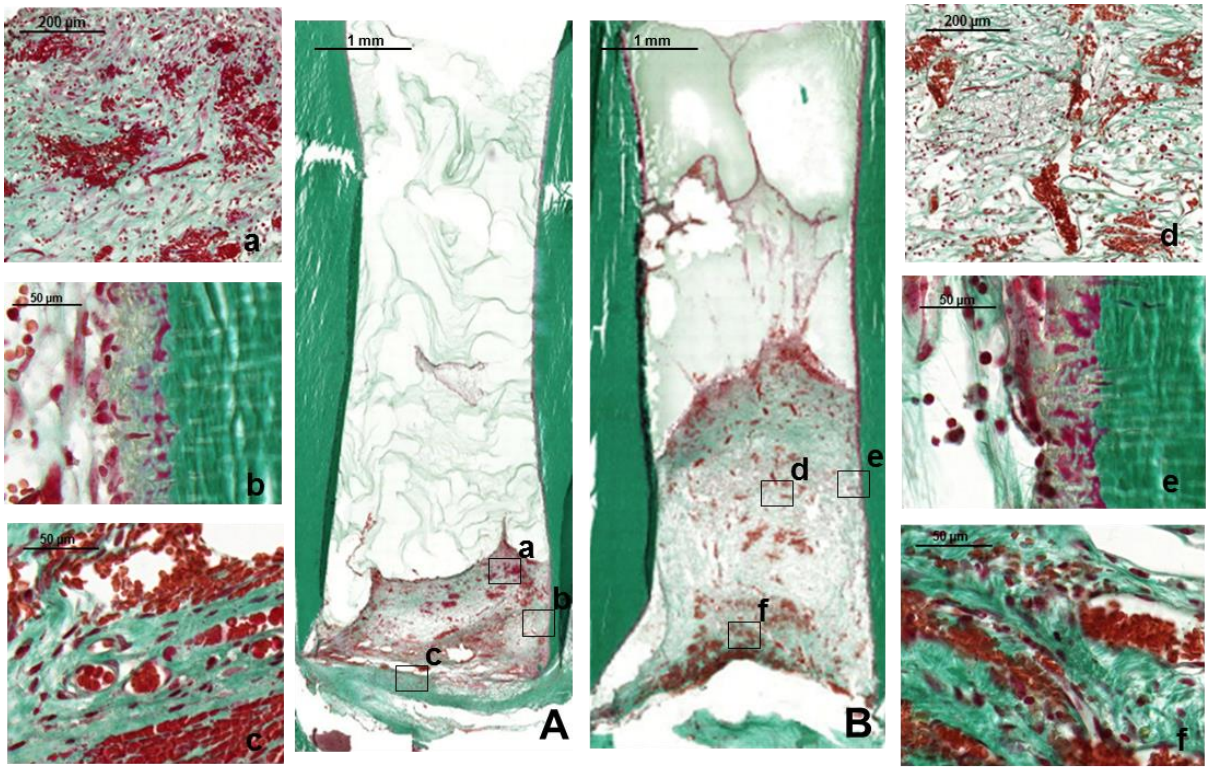

Fig. 3 Images at 6 weeks. The bilateral teeth were filled with fibrin gel that was either doped with SCF or not, and were transplanted in the same rat**. A)** Root canal filled with fibrin gel alone demonstrated tissue formation in the apical third. (a) Blood cells were evenly distributed in the newly formed tissue, and (b) the immature mineral matrix (red staining) was around half of the predentin thickness. (c) Loose connective tissue was found at apical opening. **B)** In contrast, ingrowth to the middle third of the root canal length could be identified in the pulp space filled with SCF-doped fibrin. (d) Regenerated tissue had begun to form vessel structure and (e) at the dentinal wall showed a larger area of newly formed mineral matrix. (f) The root apex was covered by connective tissue supplemented with abundant blood vessels.

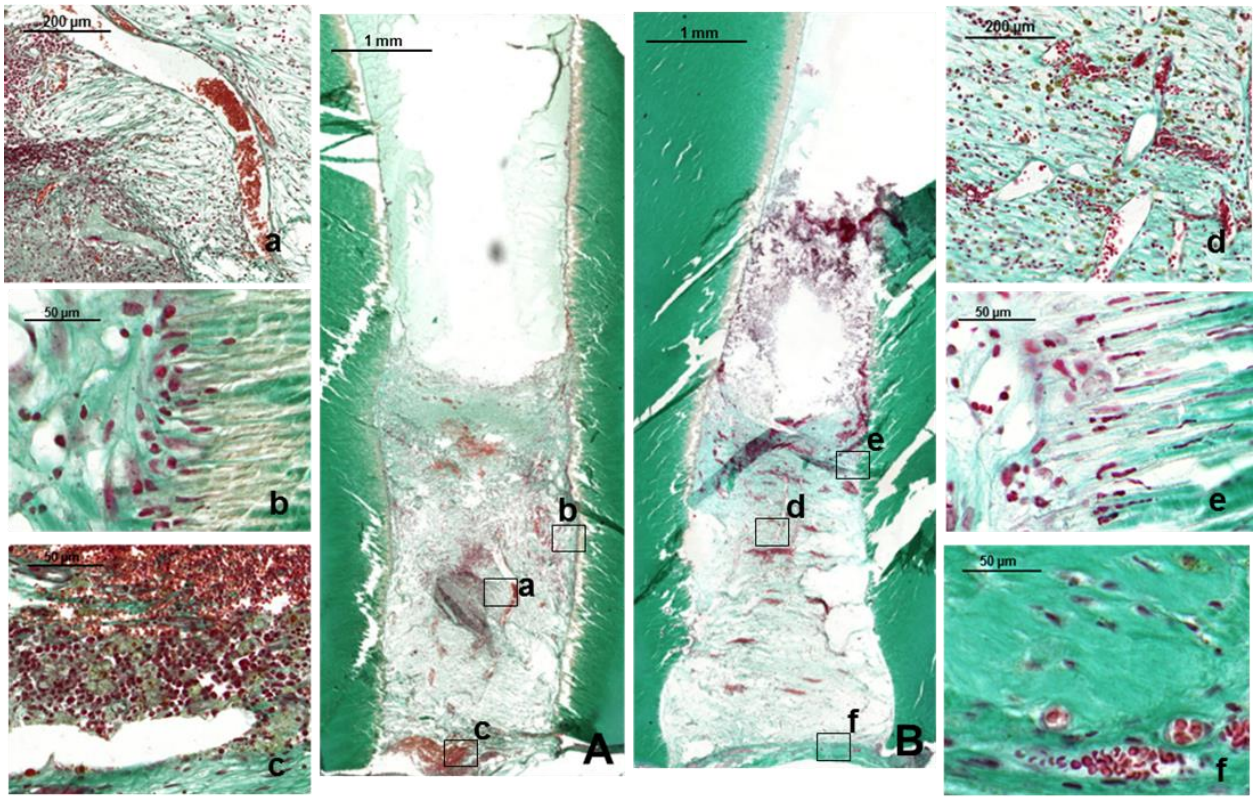

Fig. 4 Histological observations at 12 weeks. Root canals filled with the SCF-combined fibrin gel were placed on top of rat calvaria together with a paired tooth filled with mere fibrin gel as a control **A)** Fibrin gel only. Tissue ingrowth has occurred up to the middle third. (a) Vessel structures showed in the newly formed tissue and (b) odontoblast-like cells extended their cell process into dentinal tubules. (c) At the apical area, a lot of cells were located within the connective tissue. **B)** Fibrin gel with SCF. Tissue ingrowth had occurred over two-thirds of the root canal and (d) appeared highly vascularized. (e) Odontoblast-like cells were lined at the wall; odontoblastic processes were longer compared to the fibrin only group. (f) In the apical opening, a highly dense connective tissue resembling mineralized tissue had formed.

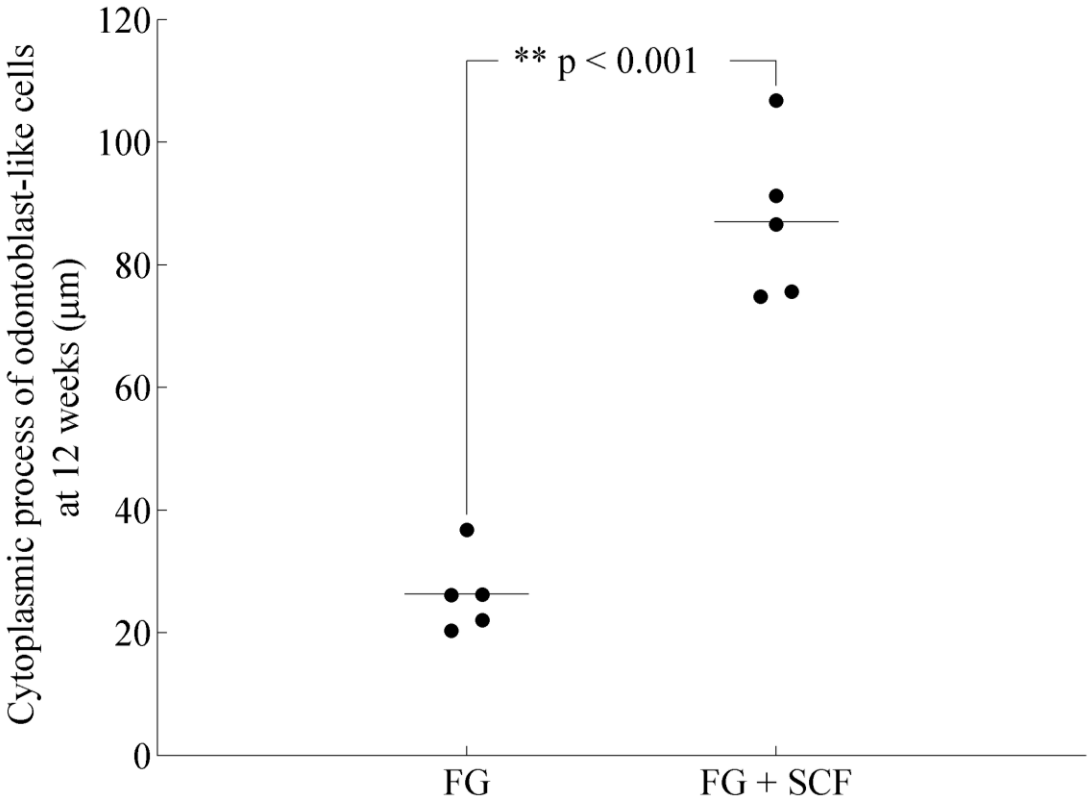

Fig. 5 Odontoblastic process measurement. The maturation of odontoblast-like cells in the 12-week specimens of SCF loaded fibrin gel was more pronounced since the cytoplasmic processes reached significantly deeper into the tubular dentin. FG: Fibrin gel, FG+SCF: Fibrin gel with SCF

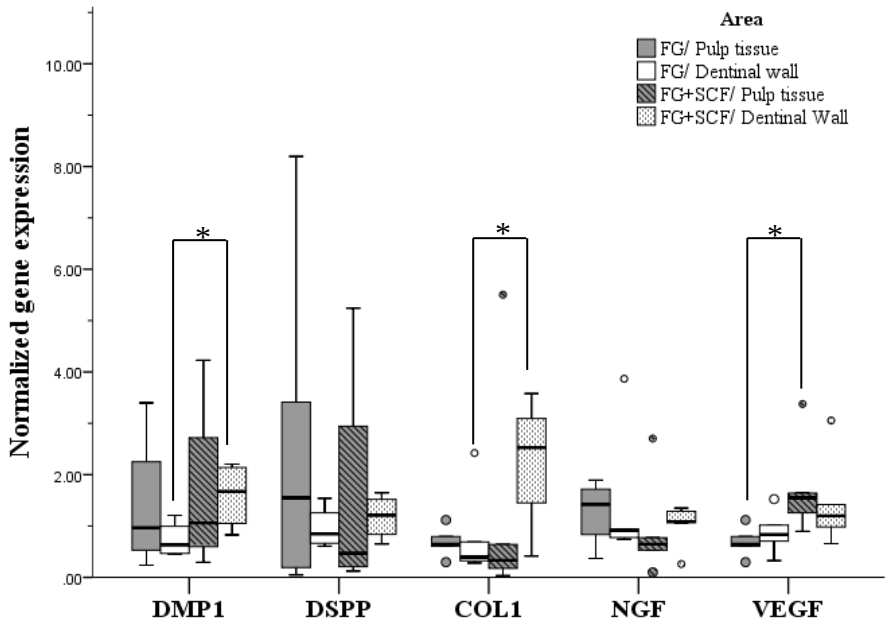

Fig. 6 Normalized expressions of mRNA for DMP1, DSPP, COL1, NGF and VEGF at 12 weeks. SCF increased DMP1 and COL1 expression in cells attached to the dentinal wall (*P < 0.05 compared with fibrin gel alone by Mann-Whitney test) and upregulated VEGF expression in the tissue ingrowth while the others did not differ by statistical comparison. FG: Fibrin gel, FG+SCF: Fibrin gel with SCF

4.4 Discussion

SCF is a chemokine shown to promote migration, neovascularization, and collagen remodeling of dental pulp progenitor cells [21]. To address its potential to improve current cell homing strategies, we tested the effect of SCF on the directionality of hMSCs migration *in vitro* and tested its *in vivo* impact by delivering it with the fibrin gel in a cell homing model [10].

Our results showed that SCF directs migration of mesenchymal stem cells but has a minor effect on their proliferation and differentiation. The concentration of SCF needed to direct the migration of MSCs was higher than previously reported [21, 28]. This might be due to the methodology used to evaluate cell migration and/or the supplier of the SCF. The advantage of the current methodology is that chemotaxis slides allow observing the migration in linear and stable concentration profiles, and to distinguish between random migration from primed and directed migration [23]. As the time frame for proliferation was longer than the time frame for migration, prolonged exposure of a high SCF concentration directly on hMSCs appeared to cause cell distress [20] and not an increased hMSC proliferation. In fact, our finding showed a responsive dose of SCF to promote cell proliferation similar to the effective dose of recent studies [21, 29] although their observation was in a longer period and in a different condition growth medium. Furthermore, the proliferative effect by SCF was observed higher than by FGF, which is a common growth factor used for hMSCs culture [22]. Beyond proliferation, high SCF concentrations affected the osteogenic differentiation since ALP activity was also increased, as in the previous studies [20, 21] where SCF was found to increase the expression of Collagen type I and III, basic fibroblast growth factor, bone morphogenic protein 6 and tissue inhibitor of metalloproteinase 1 and 4 in MSCs. All these proteins are involved in proliferation, chondrogenesis and extracellular matrix protein regulation [20].

In our *in vivo* model, we saw an effect of SCF on cell ingrowth into the pulp space after 6 weeks. On the molecular level SCF can stimulate the signaling cascades of PI3K/Akt and MEK/ERK, thereby rapidly increase ERK and AKT phosphorylation within the cells [21]. Such very early effects can be the reason why at 6 weeks we still see an increase in tissue ingrowth in fibrin gels with SCF compared to fibrin gels alone. Therefore, SCF accelerates cells homing in the pulp space. At 12 weeks, however, the difference in tissue ingrowth was not significantly increased with SCF compared to the pulps filled with mere fibrin gels. For both time points, the amount of regenerated tissue was similar to an earlier study in which tissue regeneration was achieved by stem cell delivery in immature dog teeth at 14 days and 28 days respectively [30, 31]. This would imply that the use of SCF for cell homing accelerated tissue ingrowth, yet a further acceleration can only be achieved by the application of stem cells. Compared to the application of the revascularization technique in rat teeth at the same period [32], fibrin gels with SCF apparently resulted in more tissue ingrowth. However, these results were obtained in different models and can thus not be compared conclusively.

At 12 weeks the addition of SCF did not yield in a significant improvement of tissue ingrowth. The tissue morphology at 12 weeks with SCF resembled the outcome observed with fibrin only [10]. Nevertheless, more calcified tissue could be found at the apical opening, and odontoblastic processes extended further into the dentinal tubules in teeth with SCF. Together, these results suggest that under the influence of SCF odontoblast-like cells formed earlier, covered more of the pulp and extended further into dentin [33]. Therefore, SCF secondarily accelerates the maturation of the newly formed pulp tissue and the overall regeneration process.

Evolvement in tissue maturation by SCF is also evident in the gene expression analysis. As a result of SCF application, COL1 and DMP1 were upregulated in cells adhering to dentin

where the migrated cells should be transformed to odontoblasts and hence produce minerals. The role of SCF to stimulate collagen organization of mineral matrix through the upregulation of COL I and III was already recognized [21], whereas the effect on DMP1 expression, has yet not been described and therefore introduces other roles of SCF during dentinogenesis. Since DMP1 regulates DSPP transcription during early odontoblast maturation and function [34], in our tissue, the odontoblasts may have been in different maturation stages, where DSPP gene was not increased yet. Nevertheless, this impact of SCF on genes regulating odontoblast maturation and function supports our result present *in vitro*. SCF also showed to establish a pro-angiogenic milieu by increasing VEGF upregulation in the recruited cells of the in-grown tissue (Fig.6) and exhibiting abundant blood vessels (Fig 4), similar to what was reported earlier for triggering revascularization of infarcted tissue [35, 36]. However, NGF expression did not differ which is in line with histology where we did not see an increased re-innervation.

In conclusion, SCF is able to regulate stem cell localization, proliferation and differentiation. It also increases tissue ingrowth at 6 weeks in addition to the formation of a better developed pulp-like tissue at 12 weeks. Thus, in conclusion and under the conditions of the current study, the cell homing process was accelerated by the application of SCF.

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Chapter 5

General discussion, conclusion and outlook

In the last two decades, pulp repair/regeneration of immature permanent teeth mainly relied on a revascularization technique, meant to deliver host stem cells from the apical papilla by provoked bleeding. Instead of using a scaffold and growth factors, as was suggested in the cell homing concept, the blood clot is the matrix for cells to home while the growth factors are deposited during the coagulation cascade and derive from the platelets [1]. The high variability of the outcome by the application of the revascularization technique makes it difficult to control and foresee the result. A functional cell homing concept is thus desirable to replace the revascularization technique currently performed in clinics. To this end, a suitable scaffold material modulated by growth factors is required to increase the predictability of the outcome. A fibrin gel is able to substitute the bleeding, since fibrin is the main component of a blood clot, while root canal irrigants, especially EDTA, can re-expose or release intrinsic growth factors embedded in dentin [2, 3]. Cell homing in a fibrin gel still differs from blood infiltration by the lack of platelets, which serve as one source for growth factors. Also, a synthetic scaffold material such as the fibrin gel lacks the cells derived from the apical papilla or other MSCs that are present in the blood clot during the infiltration process [4]. To study the cell homing concept in greater detail, we established a reliable and controllable animal model of clinical relevance.

In our cell homing model, human teeth are placed subcutaneously on top of rat calvaria, and can be used efficiently and conveniently to perform experiments at a rather high number. The

model is simple in terms of tooth specimen preparation and overall operation procedure. It allows the use of human teeth, and causes less traumatic pain to the animal than performing this kind of research with animal's teeth [5]. The teeth positioned on the bone also mimic a more the adequate clinical environment compared to placing the specimens at the dorsum [6]. The embedding of teeth close to the calvarial bone resembles the bony site in the dental alveolus (tooth socket). Moreover, the advantage of our model in comparison to placing the teeth at the animal dorsum was substantiated by the fact that more tissue and more mature pulp-like tissue formed. This suggests that stem cells from the bone or the periosteum favor pulp regeneration. However, there are still some limitations. Firstly, the subcutaneous space at the calvaria is inadequate for larger specimens, such as multi-rooted human teeth. Thus, only premolars and sectioned root fragments can be implanted. Secondly, the routine self-cleaning of rat, by scratching itself at the skin of the surgical area, may lead to teeth displacement. This, however, can be overcome by careful surgical technique, wound closure and a proper alignment of the specimens.

The characterization of MSCs producing pulp-like tissue can explain the biological process behind the cell homing concept. Cell homing in the current approach showed to direct MSCs into the fibrin gel. This is in line with the clinical observation of the revascularization technique, implying that (stem) cell delivery from other sources is not necessary [7]. In our model, we did not identify a specific stem cell source besides the advantage of placing the specimens close to the calvarial bone. At very early time points, we found equal numbers of MSCs in the pulpal space of teeth and in the lumen of silicon tubes, suggesting that MSC-like cells migrate alike into the fibrin gels enclosed by dentin or silicone. Maturation into odontoblast-like cells, however, occurs solely in the pulp space and depends on the surface-cues provided by dentin and the factors released from the dentinal wall.

The cell homing approach seems to provide sufficient elements for supporting pulp tissue regeneration. The overall survival of the developing tooth, however, will depend on the velocity of regeneration and maturation. To that end, we tested the stem cell factor (SCF) for its potential to accelerate this regenerative process. SCF was selected because it is known to recruit autologous stem cells to repair damaged organs [8]. In addition, it was shown that SCF is able to direct human mesenchymal stem cells (hMSCs) migration, and increase the number of $CD105^+$, $CD90^+$ and $CD45^-$ cells even in silicon tubes (Appendix A). In addition, SCF stimulated cell proliferation and differentiation *in vitro*. *In vivo*, we also found that SCF supported tissue maturation, based on larger areas of tissue ingrowth at 6 weeks and pulp-like tissue regeneration including longer odontoblastic processes and upregulation of DMP1, COL1 and VEGF at 12 weeks. Compared to other chemokines used for cell homing during pulp regeneration, SCF can be applied alone and accelerate pulp-like tissue maturation in the root canals to the full length of immature permanent teeth [9, 10]. However, the cell homing process enhanced by SCF appears to still not be equivalent to stem cell transplantation [11, 12].

This study suggests the application of the cell homing concept in combination with SCF in pulp regeneration is simple, reasonable, effective and comparable or even superior to the clinically applied revascularization method. In the future, clinical trials have to provide the data to test this experimental treatment *in situ*.

5.1 Outlook

The goal of cell homing research in pulp regeneration is the development of a new treatment option, which should be transferrable to clinical practice in the foreseeable future. The use of a fibrin gel together with intrinsic growth factors released from dentin to create an autologous dental pulp construct has the potential to overcome the drawbacks of the revascularization

technique. In order to meet these expectations, the nature of the ideal biological milieu for conditioning the autologous pulp will need to be complemented. In addition, standardized criteria to quantitatively evaluate the success tissue-engineered dental pulps, as well as noninvasive analytical techniques for application in animal models and patients, will be required. In the future this technique or an adjusted version could even prove useful for the treatment of mature teeth.

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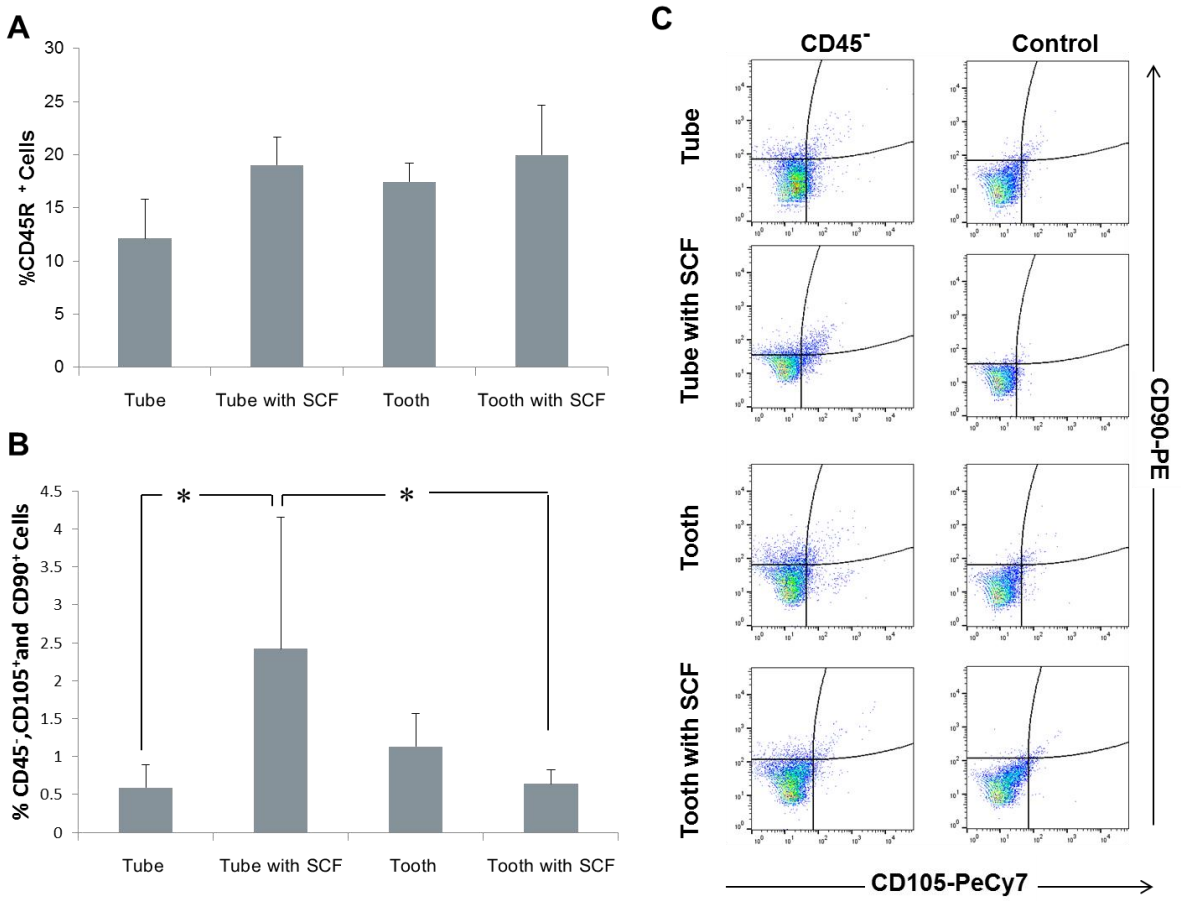
Appendix A



Flow cytometry analysis of the isolated cells from four groups, including human teeth with fibrin gel +/- SCF and silicone tubes with fibrin gel +/- SCF, which serve as a control. A) Amount of CD45R positive cells was analyzed. No significant difference was shown between groups. B) After the exclusion of $CD45R^{+}$cells, the number of CD105 and CD90 co-expression was investigated. SCF treated fibrin gel in the silicone tubes significantly contained higher amount of cells with MSCs phenotype markers ($CD45R^{-}$, $CD105^{+}$ and $CD90^{+}$) compared to the non-treated silicone tubes and SCF-treated teeth (* $P \leq 0.05$). C) The evidence was quite low as shown in the pseudo plots for CD105 and CD90 expression.

List of Abbreviation

DPP	Dentin phosphoprotein
DSP	Dentin sialoprotein
OCN	Osteonectin
BSP	Bone sialoprotein
BMP2	Bone morphogenic protein 2
TGF-β	Trans forming growth factor –β
bFGF/FGF2	Basic fibroblast growth factor
TNF-α	Tumor necrosis factor-α
SDF-1	Stromal-derived factor -1
CXCR4	C-X-C chemokine receptor type 4
SCF	Stem cell factor
c-kit	Tyrosine-protein kinase kit
p38-MAPK	P38 mitogen-activated protein kinases
MTA	Mineral trioxide aggregate
EDTA	Ethylenediaminetetraacetic acid
MSCs	Mesenchymal stem cells
DPSCs	Dental pulp stem cells
BMSCs	Bone marrow stem cells
ADPCs	Adipose-derived stem cells
SCAP	Stem cells from apical papilla
FDA	Food and drug administration

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Research Experience:

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